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(54) **REGION DE REGULATION DE L'ADN POUR LA PEROXYDASE  
DES TEGUMENTS**

(54) **SEED COAT DNA REGULATORY REGION AND PEROXIDASE**

(57) Caractérisation et présentation d'une nouvelle séquence génomique spécifique pour le tégument. Les régions régulatrices voisines de l'ADN ont également été caractérisées. Le peroxydase de tégument est traduit sous forme de protéine précurseur de 38 kDa, a 352 acides aminés, renfermant une séquence-signal de 26 acides aminés, elle donne, par clivage, une protéine de 35 kDa. Les plantes renfermant un allèle Ep dominant accumulent de grandes quantités de peroxydase dans les cellules sabliers du subépiderme. Les génotypes epep homozygotes récessifs n'accumulent pas de peroxydase dans ces cellules et leur part dans l'activité totale de la peroxydase du tégument se trouve sensiblement réduite. Les sondes dérivées de l'ADNc ou de l'ADN génomique peuvent servir à sélectionner les polymorphismes qui distinguent les génotypes EpEp et epep. La cosegregation des polymorphismes dans une population F<sub>2</sub> provenant d'un croisement de plantes EpEp et epep montre que le locus Ep code la protéine peroxydase. Une comparaison des allèles Ep et ep révèle qu'il manque 87 bp dans le gène récessif pour le codon initial de traduction. L'expression hétérologue ainsi que les vecteurs et les hôtes utilisés pour l'expression de la peroxydase du tégument sont également présentés. La région régulatrice de l'ADN spécifique pour la semence peut servir à contrôler l'expression i) de certains gènes, comme ceux codant la résistance aux herbicides, ii) de protéines virales du tégument, protégeant contre l'infection, iii) de protéines à intérêt commercial (p. ex. en pharmacie), iv) de protéines modifiant la valeur nutritive, le goût ou le conditionnement des semences, enfin, elle peut servir à v) éliminer biologiquement des insectes ou des agents pathogènes (p. ex. B. thuringiensis).

(57) A novel seed coat specific peroxidase genomic sequence is characterized and presented. Adjacent DNA regulatory regions have also been characterized. The seed coat peroxidase is translated as a 352 amino acid precursor protein of 38 kDa comprising a 26 amino acid signal sequence which when cleaved results in a 35 kDa protein. Plants containing a dominant Ep allele accumulate large amounts of peroxidase in the hourglass cells of the subepidermis. Homozygous recessive epep genotypes do not accumulate peroxidase in the hourglass cells and are much reduced in total seed coat peroxidase activity. Probes derived from the cDNA, or genomic DNA can be used to detect polymorphisms that distinguished EpEp and epep genotypes. Cosegregation of the polymorphisms in an F<sub>2</sub> population from a cross of EpEp and epep plants shows that the Ep locus encodes the seed coat peroxidase protein. Comparison of Ep and ep alleles indicates that the recessive gene lacks 87 bp of sequence encompassing the translation start codon. The heterologous expression, as well as vectors and hosts to be used for the expression of the seed coat peroxidase, are also disclosed. The seed-specific DNA regulatory region may be used to control expression of genes of interest such as i) genes encoding herbicide resistance, or ii) biological control of insects or pathogens (e.g. B. thuringiensis), or iii) viral coat proteins to protect against viral infections, or iv) proteins of commercial interest (e.g. pharmaceutical), and v) proteins that alter the nutritive value, taste, or processing of seeds.



### ABSTRACT OF THE DISCLOSURE

A novel seed coat specific peroxidase genomic sequence is characterized and presented. Adjacent DNA regulatory regions have also been characterized. The seed coat peroxidase is translated as a 352 amino acid precursor protein of 38 kDa comprising a 26 amino acid signal sequence which when cleaved results in a 35 kDa protein. Plants containing a dominant *Ep* allele accumulate large amounts of peroxidase in the hourglass cells of the subepidermis. Homozygous recessive *epep* genotypes do not accumulate peroxidase in the hourglass cells and are much reduced in total seed coat peroxidase activity. Probes derived from the cDNA, or genomic DNA can be used to detect polymorphisms that distinguished *EpEp* and *epep* genotypes. Cosegregation of the polymorphisms in an  $F_2$  population from a cross of *EpEp* and *epep* plants shows that the *Ep* locus encodes the seed coat peroxidase protein. Comparison of *Ep* and *ep* alleles indicates that the recessive gene lacks 87 bp of sequence encompassing the translation start codon. The heterologous expression, as well as vectors and hosts to be used for the expression of the seed coat peroxidase, are also disclosed. The seed-specific DNA regulatory region may be used to control expression of genes of interest such as i) genes encoding herbicide resistance, or ii) biological control of insects or pathogens (e.g. *B. thuringiensis*), or iii) viral coat proteins to protect against viral infections, or iv) proteins of commercial interest (e.g. pharmaceutical), and v) proteins that alter the nutritive value, taste, or processing of seeds.

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**SEED COAT SPECIFIC DNA REGULATORY REGION AND PEROXIDASE**

The present invention relates to a novel DNA molecule comprising a plant seed coat specific DNA regulatory region and a novel structural gene encoding a peroxidase. The seed-coat specific DNA regulatory region may also be used to control the expression of other genes of interest within the seed coat.

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**BACKGROUND OF THE INVENTION**

Full citations for references appear at the end of the Examples section.

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Peroxidases are enzymes catalyzing oxidative reactions that use  $H_2O_2$  as an electron acceptor. These enzymes are widespread and occur ubiquitously in plants as isozymes that may be distinguished by their isoelectric points. Plant peroxidases contribute to the structural integrity of cell walls by functioning in lignin biosynthesis and suberization, and by forming covalent cross-linkages between extension, cellulose, pectin and other cell wall constituents (Campa, 1991). Peroxidases are also associated with plant defence responses and resistance to pathogens (Bowles, 1990; Moerschbacher 1992). Soybeans contain 3 anionic isozymes of peroxidase with a minimum  $M_r$  of 37 kDa (Sessa and Anderson, 1981). Recently one peroxidase isozyme, localised within the seed coat of soybean, has been characterized with a  $M_r$  of 37 kDa (Gillikin and Graham, 1991).

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In an analysis of soybean seeds, Buttery and Buzzell (1968) showed that the amount of peroxidase activity present in seed coats may vary substantially among different cultivars. The presence of a single dominant gene *Ep* causes a high seed coat peroxidase phenotype (Buzzell and Buttery, 1969). Homozygous recessive *epep* plants are ~100-fold lower in seed coat peroxidase activity. This results from a reduction in the amount of peroxidase enzyme present, primarily in the hourglass cells of the subepidermis (Gijzen *et al.*, 1993). In plants carrying the *Ep* gene, peroxidase is heavily concentrated in the hourglass cells (osteosclereids). These cells form a highly differentiated cell layer with thick, elongated secondary walls and large intercellular spaces (Baker *et al.*, 1987). Hourglass cells develop between the epidermal macrosclereids and the underlying articulated parenchyma, and are a prominent feature of seed coat anatomy at full maturity. The cytoplasm exudes from the hourglass cells upon imbibition with water and a distinct peroxidase isozyme constitutes five to 10% of the total soluble protein in *EpEp* seed coats. It is not known why the hourglass cells accumulate large amounts of peroxidase, but the sheer abundance and relative purity of the enzyme in soybean seed coats is significant because peroxidases are versatile enzymes with many commercial and industrial applications. Studies of soybean seed coat peroxidase have shown this enzyme to have useful catalytic properties and a high degree of thermal stability even at extremes of pH (McEldoon *et al.*, 1995). These properties result in the preferred use of soybean peroxidase, over that of horseradish peroxidase, in diagnostic assays as an enzyme label for antigens, antibodies, oligonucleotide probes, and within staining techniques. Johnson *et al* report on the use of soybean peroxidase for the deinking of printed waste paper (U.S. 5,270,770;

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December 6, 1994) and for the biocatalytic oxidation of primary alcohols (U.S. 5,391,488; February 13, 1996). Soybean peroxidase has also been used as a replacement for chlorine in the pulp and paper industry, or as formaldehyde replacement (Freiberg, 1995).

5       An anionic soybean peroxidase from seed coats has been purified (Gillikin and Graham, 1991). This protein has a pI of 4.1 and  $M_r$  of 37 kDa. A method for the bulk extraction of peroxidase from seed hulls of soybean using a freeze thaw technique has also been reported (U.S. 5,491,085, February 13, 1996, Pokara and Johnson).

10       Lagrimini et al (1987) disclose the cloning of a ubiquitous anionic peroxidase in tobacco encoding a protein of  $M_r$  of 36 kDa. This peroxidase has also been over expressed in transgenic tobacco plants (Lagrimini et al 1990) and Maliyakal discloses the expression of this gene in cotton (WO 95/08914).

15       Huangpu et al (1995) reported the partial cloning of a soybean anionic seed coat peroxidase. The 1031 bp sequence contained an open reading frame of 849 bp encoding a 283 amino acid protein with a  $M_r$  of 30,577. The  $M_r$  of this peroxidase is 7 kDa less than what one would expect for a soybean seed coat peroxidase as reported by Gillikin and Graham (1991) and possibly represents another peroxidase isozyme  
20       within the seed coat.

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The upstream promoter sequences for two poplar peroxidases have been described by Osakabe et al (1995). A number of characteristic regulatory sites were identified from comparison of these sequences to existing promoter elements. Additionally, a cryptic promoter with apparent specificity for seed coat tissues was isolated from tobacco by a promoter trapping strategy (Fobert et al. 1994). The upstream regulatory sequences associated with the Ep gene in soybean are distinct from these and other previously characterized promoters. The soybean Ep promoter drives high-level expression in a cell and tissue specific manner. The peroxidase protein encoded by the Ep gene accumulates in the seed coat tissues, especially in the hour glass cells of the subepidermis. Minimal expression of the gene is detected in root tissues.

One problem arising from the desired use of soybean seed coat peroxidase is that there is variability between soybean varieties regarding peroxidase production (Buttery and Buzzell, 1986; Freiberg, 1995). Due to the commercial interest in the use of soybean seed coat peroxidase new methods of producing this enzyme are required. Therefore, the gene responsible for the expression of the 37 kDa isozyme in soybean seed coat was isolated and characterized.

Furthermore, novel regulatory regions obtained from the genomic DNA of soybean seed coat peroxidase have been isolated and characterized and are useful in directing the expression of genes of interest in seed coat tissues.

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**SUMMARY OF THE INVENTION**

The present invention relates to a DNA molecule that encodes a soybean seed coat peroxidase and associated DNA regulatory regions.

This invention also embraces isolated DNA molecules comprising the nucleotide  
5 sequence of either SEQ ID NO:1 (the cDNA encoding soybean seed coat peroxidase)  
SEQ ID No:2 (the genomic sequence).

This invention also provides for a chimeric DNA molecule comprising a seed  
coat-specific regulatory region having nucleotides 1-1532 of SEQ ID NO:2 and a gene  
10 of interest under control of this DNA regulatory region. Also included within this  
invention are chimeric DNA molecules comprising genomic DNA sequences  
exemplified by nucleotides 412-1041, 1234-2263 or 2430-2691 of SEQ ID NO:2.  
Furthermore, this invention is directed to isolated DNA molecules comprising at least  
1) 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID  
15 NO:2;  
2) 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ  
ID NO:2;  
3) 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ  
ID NO:2; or  
20 4) 22 contiguous nucleotides selected from nucleotides 2430-2691 of SEQ  
ID NO:2.

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The present invention also provides for vectors which comprise DNA molecules encoding soybean seed coat peroxidase. Such a construct may include the DNA regulatory region from SEQ ID NO:2, including nucleotides 1-1532, or at least 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2 in conjunction with the seed coat peroxidase gene, or the seed coat peroxidase gene under  
5 the control of any suitable constitutive or inducible promoter of interest.

This invention is also directed towards vectors which comprise a gene of interest placed under the control of a DNA regulatory element derived from the genomic sequence encoding soybean seed coat peroxidase. Such a regulatory element  
10 includes nucleotides 1-1532 of SEQ ID NO:2, or at least 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2. Elements comprising nucleotides 412-1041, 1234-2263 or 2430-2691 of SEQ ID NO:2, or 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ ID NO:2, 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ ID NO:2, or 22 contiguous nucleotides  
15 selected from nucleotides 2430-2691 of SEQ ID NO:2 may also be used.

This invention also embraces prokaryotic and eukaryotic cells comprising the vectors identified above. Such cells may include bacterial, insect, mammalian, and plant cell cultures.  
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This invention also provides for transgenic plants comprising the seed coat peroxidase gene under control of constitutive or inducible promoters. Furthermore,



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this invention also relates to transgenic plants comprising the DNA regulatory regions of nucleotides 1-1532 of SEQ ID NO:2 controlling a gene of interest, or comprising genes of interest in functional association with genomic DNA sequences exemplified by nucleotides 412-1041, 1234-2263 or 2430-2691 of SEQ ID NO:2. Also embraced by this invention are transgenic plants having regulatory regions comprising at least 24  
5 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2, 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ ID NO:2, 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ ID NO:2, or 22 contiguous nucleotides selected from nucleotides 2430-2691 of SEQ ID NO:2.

10 This invention is also directed to a method for the production of soybean seed coat peroxidase in a host cell comprising:

- i) transforming the host cell with a vector comprising an oligonucleotide sequence that encodes soybean seed coat peroxidase; and
- ii) culturing the host cell under conditions to allow expression of the  
15 soybean seed coat peroxidase.

This invention also provides for a process for producing a heterologous gene of interest within seed coats of a transformed plant, comprising propagating a plant transformed with a vector comprising a gene of interest under the control of  
20 nucleotides 1-1532 of SEQ ID NO:2. Furthermore, this invention embraces a process for producing a heterologous gene of interest within seed coats of a transformed plant, comprising propagating a plant transformed with a vector comprising a gene of interest

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under the control of a regulatory region comprising at least 24 nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2.

Although the present invention is exemplified by a soybean seed coat peroxidase and adjacent DNA regulatory regions, in practice any gene of interest can be placed  
5 downstream from the DNA regulatory region for seed coat specific expression.

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**BRIEF DESCRIPTION OF THE DRAWINGS**

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

Figure 1 is the cDNA and deduced amino acid sequence of soybean seed coat peroxidase. Nucleotides are numbered by assigning +1 to the first base of the ATG start codon; amino acids are numbered by assigning +1 to the N-terminal Gln residue after cleavage of the putative signal sequence. The N-terminal signal sequence, the region of the active site, and the heme-binding domain are underlined. The numerals I, II and III placed directly above single nucleotide gaps in the sequence indicate the three intron splice positions. The target site and direction of five different PCR primers are shown with dotted lines above the nucleotide sequence. An asterisk (\*) marks the translation stop codon.

Figure 2 is the genomic DNA sequence of the Soybean seed coat peroxidase.

Figure 3 is a comparison of soybean seed coat peroxidase with other closely related plant peroxidases. The GenBank accession numbers are provided next to the name of the plant from which the peroxidase was isolated. The accession number for the soybean sequence is L78163. (A) A comparison of the nucleic acid sequences; (B) A comparison of the amino acid sequences.

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Figure 4 is a restriction fragment length polymorphisms between *EpEp* and *epep* genotypes using the seed coat peroxidase cDNA as probe. Genomic DNA of soybean lines OX312 (*epep*) and OX347 (*EpEp*) was digested with restriction enzyme, separated by electrophoresis in a 0.5% agarose gel, transferred to nylon, and hybridized with <sup>32</sup>P-labelled cDNA encoding the seed coat peroxidase. The size of the hybridizing fragments was estimated by comparison to standards and is indicated on the right.

Figure 5 exhibits the structure of the *Ep* Locus. A 17 kb fragment including the *Ep* locus is illustrated schematically. A 3.3 kb portion of the gene is enlarged and exons and introns are represented by shaded and open boxes, respectively. The final enlargement of the 5' region shows the location and DNA sequence around the 87 bp deletion occurring in the *ep* allele of soybean line OX312. Nucleotides are numbered by assigning +1 to the first base of the ATG start codon.

Figure 6 displays PCR analysis of *EpEp* and *epep* genotypes using primers derived from the seed coat peroxidase cDNA. Genomic DNA from soybean lines OX312 (*epep*) and OX347 (*EpEp*) was used as template for PCR analysis with four different primer sets. Amplification products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. Genotype and primer combinations are

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indicated at the top of the figure. The size in base pairs of the amplified DNA fragments are indicated on the right.

Figure 7 exhibits PCR analysis of an F<sub>2</sub> population from a cross of *EpEp* and *epep* genotypes. Genomic DNA was used as template for PCR analysis of the parents (P) and 30 F<sub>2</sub> individuals. The cross was derived from the soybean lines OX312 (*epep*) and OX347 (*EpEp*). Plants were self pollinated and seeds were collected and scored for seed coat peroxidase activity. The symbols (-) and (+) indicate low and high seed coat peroxidase activity, respectively. Primers prx9+ and prx10- were used in the amplification reactions. Products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. The migration of molecular markers and their corresponding size in kb is also shown (lanes M).

Figure 8 displays PCR analysis of six different soybean cultivars with primers derived from the seed coat peroxidase cDNA sequence. Genomic DNA was used as template for PCR analysis of three *EpEp* cultivars and three *epep* cultivars. Primers used in the amplification reactions and the size of the DNA product is indicated on the left. Products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide.

(A) Forward and reverse primers are downstream from deletion

(B) Forward primer anneals to site within deletion

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## (C) Primers span deletion

**Figure 9** shows the accumulation of peroxidase RNA in tissues of GEp and *epep* plants. **Figure 9(A):** A comparison of peroxidase transcript abundance in cultivars Harosoy 63 (Ep) or Marathon (ep). Seed and pod tissues were sampled at a late stage of development corresponding to a whole seed fresh weight of 250 mg. Root and leaf tissue was from six week old plants. Autoradiograph exposed for 96 h. **Figure 9(B):** Developmental expression of peroxidase in cultivar Harosoy 63 (Ep). Flowers were sampled immediately after opening. Seed coat tissues were sampled at four stages of development corresponding to a whole seed fresh weight of: lane 1, 50 mg; lane 2, 100 mg; lane 3, 200 mg; lane 4, 250 mg. Autoradiograph exposed for 20 h.

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**DESCRIPTION OF PREFERRED EMBODIMENT**

The present invention is directed to a novel oligonucleotide sequence encoding a seed coat peroxidase and associated DNA regulatory regions.

According to the present invention DNA sequences that are "substantially homologous" includes sequences that are identified under conditions of high stringency. "High stringency" refers to Southern hybridization conditions employing washes at 65°C with 0.1 x SSC, 0.5 % SDS.

By "DNA regulatory region" it is meant any region within a genomic sequence that has the property of controlling the expression of a DNA sequence that is operably linked with the regulatory region. Such regulatory regions may include promoter or enhancer regions, and other regulatory elements recognized by one of skill in the art. A segment of the DNA regulatory region is exemplified in this invention, however, as is understood by one of skill in the art, this region may be used as a probe to identify surrounding regions involved in the regulation of adjacent DNA, and such surrounding regions are also included within the scope of this invention.

In the context of this disclosure, the term "promoter" or "promoter region" refers to a sequence of DNA, usually upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site.

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There are generally two types of promoters, inducible and constitutive. An "inducible promoter" is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible promoter to activate transcription, is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

By "constitutive promoter" it is meant a promoter that directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive promoters include those associated with the CaMV 35S transcript and *Agrobacterium* Ti plasmid nopaline synthase gene.

The chimeric gene constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The



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polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

5           Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumour inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene  
10 of the present construct can therefore be used to construct chimeric genes for expression in plants.

          The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These  
15 enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the  
20 source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

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To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* ( $\beta$ -glucuronidase), or luminescence, such as luciferase are useful.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example

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Weissbach and Weissbach (1988) and Geierson and Corey (1988). The present invention further includes a suitable vector comprising the chimeric gene construct.

Buttery and Buzzell (1968) showed that the amount of peroxidase activity present in seed coats may vary substantially among different cultivars. The presence of a single dominant gene *Ep* causes a high seed coat peroxidase phenotype (Buzzell and Buttery, 1959). Homozygous recessive *epep* plants are ~100-fold lower in seed coat peroxidase activity. This results from a reduction in the amount of peroxidase enzyme present, primarily in the hourglass cells of the subepidermis (Gijzen *et al.*, 1993). In plants carrying the *Ep* gene, peroxidase is heavily concentrated in the hourglass cells (osteosclereids). These cells form a highly differentiated cell layer with thick, elongated secondary walls and large intercellular spaces (Baker *et al.*, 1987).

Screening a seed coat cDNA library prepared from *EpEp* plants with a degenerate primer derived from the active site domain of plant peroxidase resulted in a high frequency of positive clones. Many of these clones encode identical cDNA molecules and indicate that the corresponding mRNA is an abundant transcript in developing seed coat tissues. The sequence of the cDNA is shown in Figure 1.

Previous studies on soybean seed coat peroxidase indicated that this enzyme is heavily glycosylated and that carbohydrate contributes 18% of the mass of the apoenzyme (Gray *et al.*, 1996). The seven potential glycosylation sites identified from the amino acid sequence of the seed coat peroxidase (Figure 1) would accommodate the

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five or six N-linked glycosylation sites proposed by Gray *et al.* (1996). The heme-binding domain encompasses residues Asp161 to Phe171 and the acid-base catalysis region from Gly33 to Cys44. The two regions are highly conserved among plant peroxidases and are centred around functional histidine residues, His169 and His40. There are eight conserved cysteine residues in the mature protein that provide for four di-sulfide bridges found in other plant peroxidases and predicted from the crystal structure of peanut peroxidase (Welinder, 1992; Schuller *et al.*, 1996). Other conserved areas include residues Cys91 to Ala105 and Val119 to Leu127 that occur in or around helix D. The most divergent aspects of the seed coat peroxidase protein sequence are the carboxy- and amino-terminal regions. These sequences probably provide special targeting signals for the proper processing and delivery of the peptide chain. It is possible the carboxy-terminal extension of the seed coat peroxidase is removed at maturity, as has been shown for certain barley and horseradish peroxidases (Welinder, 1992).

The molecular mass of the enzyme has been determined by denaturing gel electrophoresis to be 37 kDa (Sease and Anderson, 1981; Gillikin and Graham, 1991) or 43 kDa (Gijzen *et al.*, 1993). Analysis by mass spectrometry indicated a mass of 40,622 Da for the apo-enzyme and 33,250 Da after deglycosylation (Gray *et al.*, 1996). These values are in good agreement with the mass of 35,377 Da calculated from the predicted amino acid sequence for the mature apo-protein prior to glycosylation and other modifications. Huangpu *et al.* (1995) reported an anionic seed coat peroxidase having a  $M_r$  of 30,577 Da and characterized a partial cDNA encoding this protein.

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This 1031 bp cDNA contained an open reading frame of 849 bp encoding a 283 amino acid protein. There are several differences between this reported sequence and the sequence of this invention that are manifest at the amino acid level (see Figure 3 for sequence comparison). The enzyme encoded by the gene reported by Huangpu et al is different from that of this invention as the peroxidase of this invention has a  $M_r$  of 35,377 Da.

Genomic DNA blots probed with the seed coat peroxidase cDNA produced two or three hybridizing fragments of varying intensity with most restriction enzyme digestions, despite that several peroxidase isozymes are present in soybean. The results indicate that this seed coat peroxidase is present as a single gene that does not share sufficient homology with most other peroxidase genes to anneal under conditions of high stringency.

The genomic DNA sequence comprises four exons spanning bp 1533-1752 (exon 1), 2383-2574 (exon 2), 3605-3769 (exon 3) and 4033-4516 (exon 4) and three introns comprising 1752-2382 (intron 1), 2575-3604 (intron 2) and 3770-4516 (intron 3), of SEQ ID NO:2. Features of the upstream regulatory region of the genomic DNA include a TATA box centred on bp 1487; a cap signal 32 bp down stream centred on bp 1520. Also noted within the genomic sequence are three polyadenylation signals centred on bp 4520, 4598, 4663 and a polyadenylation site at bp 4700.

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This promoter is considered seed coat specific since the peroxidase protein encoded by the Ep gene accumulates in the seed coat tissues, especially in the hourglass cells of the subepidermis, and is not expressed in other tissues, aside from a marginal expression of peroxidase in the root tissues. This is also true at the transcriptional level (see Figure 9). The DNA regulatory regions of the genomic sequence of Figure 2 are used to control the expression of the adjacent peroxidase gene in seed coat tissue. Such regulatory regions include nucleotides 1-1532. Other regions of interest include nucleotides 1752-2382, 2575-3604 and/or 3770-4032 of SEQ ID NO:2. Therefore other proteins of interest may be expressed in seed coat tissues by placing a gene capable of expressing the protein of interest under the control of the DNA regulatory elements of this invention. Genes of interest include but are not restricted to herbicide resistant genes, genes encoding viral coat proteins, or genes encoding proteins conferring biological control of pest or pathogens such as an insecticidal protein for example *B. thuringiensis* toxin. Other genes include those capable of the production of proteins that alter the taste of the seed and/or that affect the nutritive value of the soybean.

A modified DNA regulatory sequence may be obtained by introducing changes into the natural sequence. Such modifications can be done through techniques known to one of skill in the art such as site-directed mutagenesis, reducing the length of the regulatory region using endonucleases or exonucleases, increasing the length through the insertion of linkers or other sequences of interest. Reducing the size of DNA regulatory region may be achieved by removing 3' or 5' regions of the regulatory

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region of the natural sequence by using an endonuclease such as BAL 31 (Sambrook et al 1989). However, any such DNA regulatory region must still function as a seed coat specific DNA regulatory region.

It may be readily determined if such modified DNA regulatory elements are  
5 capable of acting in a seed coat specific manner transforming plant cells with such regulatory elements controlling the expression of a suitable marker gene, culturing these plants and determining the expression of the marker gene within the seed coat as outlined above. One may also analyze the efficacy of DNA regulatory elements by introducing constructs comprising a DNA regulatory element of interest operably  
10 linked with an appropriate marker into seed coat tissues by using particle bombardment directed to seed coat tissue and determining the degree of expression of the regulatory region as is known to one of skill in the art.

Two tandemly arranged genes encoding anionic peroxidase expressed in stems  
15 of *Populus kitakamiensis*, *prxA3a* and *prxA4a* have been cloned and characterized (Osakabe et al, 1995). Both of these genomic sequences contained four exons and three introns and encoded proteins of 347 and 343 amino acids, respectively. The two genes encode distinct isozymes with deduced  $M_r$ s of 33.9 and 34.6 kDa. Furthermore, a 532 bp promoter derived from the peroxidase gene of *Armoracia*  
20 *rusticana* has also been reported (Toyobo KK, JP 4,126,088, April 27, 1992). However, a search using GenBank revealed no substantial similarity between the promoter region, or introns 1, 2 and 3 of this invention and those within the literature.

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Digestion of the genomic DNA with *Bam*HI or *Sac*I revealed restriction fragment length polymorphisms that distinguished *EpEp* and *epep* genotypes. Although the *Xba*I digestion did not produce a readily detectable polymorphism, the size of the hybridizing fragment in both genotypes was ~14 kb. Thus, a 0.3 kb size difference is outside of the resolving power of the separation for fragments this large. Sequence analysis of *EpEp* and *epep* genotypes indicates that the mutant *ep* allele is missing 87 bp of sequence at the 5' end of the structural gene. This would account for the drastically reduced amounts of peroxidase enzyme present in seed coats of *epep* plants since the deletion includes the translation start codon and the entire N-terminal signal sequence. However, the 87 bp deletion cannot account for the differences observed in the RFLP analysis since the missing fragment does not include a *Bam*HI site and is much smaller than the 0.3 kb polymorphism detected in the *Sac*I digestion. Thus, other genetic rearrangements must occur in the vicinity of the *ep* locus that lead to these polymorphisms.

The results shown here indicate that the mutation causing low seed coat peroxidase activity occurs in the structural gene encoding the enzyme. This mutation is an 87 bp deletion in the 5' region of the gene encompassing the translation start site. Several different low peroxidase cultivars share a similar mutation in the same area, suggesting that the recessive *ep* alleles have a common origin or that the region is prone to spontaneous deletions or rearrangements.



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Due to the industrial interest in soybean seed coat peroxidase, alternate sources for the production of this enzyme are needed. The DNA of this invention, encoding the seed coat soybean peroxidase under the control of a suitable promoter and expressed within a host of interest, can be used for the preparation of recombinant soybean seed coat peroxidase enzyme.

5

Soybean seed coat peroxidase has been characterized as a lignin-type peroxidase that has industrially significant properties ie: high activity and stability under acidic conditions; exhibits wide substrate specificity; equivalent catalytic properties to that of *Phanerochaete chrysosporium* lignin peroxidase (the currently preferred enzyme used for treatment of industrial waste waters (Wick 1995) but is at least 150-fold more stable; more stable than horseradish peroxidase which is also used in industrial effluent treatments and medical diagnostic kits (McEldoon *et al.*, 1995). These properties are useful within industrial applications for the degradation of natural aromatic polymers including lignin and coal (McEldoon *et al.*, 1995), and the preferred use of soybean peroxidase, over that of horseradish peroxidase, in medical diagnostic tests as an enzyme label for antigens, antibodies, oligonucleotide probes, and within staining techniques (Wick 1995). Soybean peroxidase is also used in the deinking of printed waste paper (Johnson *et al.*, U.S. 5,270,770; December 6, 1994) and for the biocatalytic oxidation of primary alcohols (Johnson *et al.*, U.S. 5,391,488; February 13, 1996). Soybean peroxidase has also been used as a replacement for chlorine in the pulp and paper industry, in order to remove chlorine, phenolic or aromatic amine containing pollutants from industrial waste waters (Wick 1995), or as formaldehyde

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replacement (Freiberg, 1995) for use in adhesives, abrasives, and protective coatings (e.g. varnish and resins, Wick 1995).

Furthermore, the seed coat peroxidase gene may be expressed in an organ or tissue specific manner within a plant. For example, the quality and strength of cotton  
5 fiber can be improved through the over-expression of cotton or horseradish peroxidase placed under the control of a fibre-specific promoter (Maliyakal, WO 95/08914; April 6, 1995).

Similarly, seed-specific DNA regulatory regions of this invention may be used  
10 to control expression of genes of interest such as:

- i) genes encoding herbicide resistance, or
  - ii) biological control of insects or pathogens (e.g. *B. thuringiensis*), or
  - iii) viral coat proteins to protect against viral infections, or
  - iv) proteins of commercial interest (e.g. pharmaceutical), and
  - 15 v) proteins that alter the nutritive value, taste, or processing of seeds
- within the seed coat of plants.

While this invention is described in detail with particular reference to preferred  
embodiments thereof, said embodiments are offered to illustrate but not to limit the  
20 invention.

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**EXAMPLES***Plant material*

All soybean (*Glycine max* [L.] Merr) cultivars and breeding lines were from the  
 5 collection at Agriculture Canada, Harrow, Ontario.

*Seed Coat cDNA library Construction and Screening*

High seed coat peroxidase (*EpEp*) soybean cultivar Harosoy 63 plants were  
 10 grown in field plots outdoors. Pods were harvested 35 days after flowering and seeds  
 in the mid-to-late developmental stage were excised. The average fresh mass was 250  
 mg per seed. Seed coats were dissected and immediately frozen in liquid nitrogen. The  
 frozen tissue was lyophilized and total RNA extracted in 100 mM Tris-HCl pH 9.0,  
 20 mM EDTA, 4% (w/v) sarkosyl, 200 mM NaCl, and 16 mM DTT, and precipitated  
 15 with LiCl using the standard phenol/chloroform method described by Wang and  
 Vodkin (1994). The poly(A)<sup>+</sup> RNA was purified on oligo(dT) cellulose columns prior  
 to cDNA synthesis, size selection, ligation into the  $\lambda$  ZAP Express vector, and  
 packaging according to instructions (Stratagene). A degenerate oligonucleotide with the  
 5' to 3' sequence of TT(C/T)CA(C/T)GA(C/T)TG(C/T)TT(C/T)GT was synthesized  
 20 labelled to high specific activity and used as a probe to isolate peroxidase cDNA clones  
 (Sambrook *et al.*, 1989). Duplicate plaque lifts were made to nylon filters (Amersham),  
 UV fixed, and prehybridized at 36 °C for 3 h in 6 x SSC, 20 mM Na<sub>2</sub>HPO<sub>4</sub> (pH6.8),

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5 x Denhardt's, 0.4 % SDS, and 500 µg/mL salmon sperm DNA. Hybridization was in the same buffer, without Denhardt's, at 36 °C for 16 h. Filters were washed quickly with several changes of 6 x SSC and 0.1 % SDS, first at room temperature and finally at 40°C, prior to autoradiography for 16 h at -70°C with an intensifying screen.

### 5 Genomic DNA Isolation, Library Construction, and DNA Blot Analysis

Soybean genomic DNA was isolated from leaves of greenhouse grown plants or from etiolated seedlings grown in vermiculite. Plant tissue was frozen in liquid nitrogen and lyophilized before extraction and purification of DNA according to the method of Dellaporta *et al.* (1983). Restriction enzyme digestion of 30 µg DNA, separation on 0.5 % agarose gels and blotting to nylon membranes followed standard protocols (Sambrook *et al.*, 1989). For construction of the genomic library, DNA purified from Harosoy 63 leaf tissue was partially digested with *Bam*HI and ligated into the λ FIX II vector (Stratagene). Gigapack XL packaging extract (Stratagene) was used to select for inserts of 9 to 22 kb. After library amplification, duplicate plaque lifts were hybridized to cDNA probe.

Blots or filter lifts were prehybridized for 2 h at 65°C in 6 x SSC, 5 x Denhardt's, 0.5 % SDS, and 100 µg/mL salmon sperm DNA. Radiolabelled cDNA probe (20 to 50 ng) was prepared using the Ready-to-Go labelling kit (Pharmacia) and <sup>32</sup>P-dCTP (Amersham). Unincorporated <sup>32</sup>P-dCTP was removed by spin column chromatography before adding radiolabelled cDNA to the hybridization buffer

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(identical to prehybridization buffer without Denhardt's). Hybridization was for 20 h at 65°C. Membranes were washed twice for 15 min at room temperature with 2 x SSC, 0.5 % SDS, followed by two 30 min washes at 65°C with 0.1 x SSC, 0.5 % SDS. Autoradiography was for 20 h at -70°C using an intensifying screen and X-OMAT film (Kodak).

5

#### *DNA Sequencing*

Sequencing of DNA was performed using dye-labelled terminators and Taq-FS DNA polymerase (Perkin-Elmer). The PCR protocol consisted of 25 cycles of a 30 sec melt at 96°C, 15 sec annealing at 50°C, and 4 min extension at 60°C. Samples were  
10 analyzed on an Applied Biosystems 373A Stretch automated DNA sequencer.

#### *Polymerase Chain Reaction*

15 PCR amplifications contained 1 ng template DNA, 5 pmol each primer, 1.5 mM MgCl<sub>2</sub>, 0.15 mM deoxynucleotide triphosphates mix, 10 mM Tris-HCl, 50 mM KCl, pH 8.3, and 1 unit of Taq polymerase (Gibco BRL) in a total volume of 25 µL. Reactions were performed in a Perkin-Elmer 480 thermal cycler. After an initial 2 min denaturation at 94°C, there were 35 cycles of 1 min denaturation at 94°C, 1 min  
20 annealing at 52°C, and 2 min extension at 72°C. A final 7 min extension at 72°C completed the program. The following primers were used for PCR analysis of genomic DNA:

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prx2+      CTTCCAAATATCAACTCAAT  
 prx6-      TAAAGTTGGAAAAGAAAGTA  
 prx9        ATGCATGCAGGTTTTTCAGT  
 prx10-     TTGCTCGCTTTCTATTGTAT  
 prx12+     TCTTCGATGCTTCTTTCACC  
 5   prx29+    CATAACAATACGTACGTGAT

### RNA Isolation

For isolation of RNA, tissue was harvested from greenhouse grown plants,  
 10 dissected, frozen in liquid nitrogen, and lyophilized prior to extraction. Total RNA was  
 purified from seed coats, embryos, pods, leaves, and flowers using standard  
 phenol/chloroform method (Sambrook et al., 1989). This method did not afford good  
 yields of RNA from roots, therefore this tissue was extracted with Triazole reagent  
 (GibcoBRL) and total RNA purified according to manufacturers' instructions with an  
 15 additional phenol-chloroform extraction step. The amount of RNA was estimated by  
 measuring absorbance at 260 and 280 nm, and by electrophoretic separation in  
 formaldehyde gels followed by staining with ethidium bromide and comparison to  
 known standards. Total RNA (10 µg per sample) was prepared, subject to  
 electrophoresis through a 1% agarose gel containing formaldehyde, and then stained  
 20 with ethidium bromide to ensure equal loading of samples. The gel was blotted to  
 nylon (Hybond™N, Amersham) according to standard methods and the RNA was fixed  
 to the membrane by UV cross linking.

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*Seed Coat Peroxidase Assays*

The F<sub>2</sub> seed was measured for peroxidase activity to score the phenotype of the F<sub>2</sub> population because the seed testa is derived from maternal tissue. The seeds were briefly soaked in water and the seed coat was dissected from the embryo and placed in a vial. Ten drops (~500 µL) of 0.5% guaiacol was added and the sample was left to stand for 10 min before adding one drop (~50 µL) of 0.1% H<sub>2</sub>O<sub>2</sub>. An immediate change in colour of the solution, from clear to red, indicates a positive result and high seed coat peroxidase activity.

10    *Example 1: The Seed Coat Peroxidase cDNA and genomic DNA sequences*

To isolate the seed coat peroxidase transcript, a cDNA library was constructed from developing seed coat tissue of the *EpEp* cultivar Harosoy 63. The primary library contained 10<sup>6</sup> recombinant plaque forming units and was amplified prior to screening. A degenerate 17-mer oligonucleotide corresponding to the conserved active site domain of plant peroxidases was used to probe the library. In screening 10,000 plaque forming units, 12 positive clones were identified. The cDNA insert size of the clones ranged from 0.5 to 2.5 kb, but six clones shared a common insert size of 1.3 kb. These six clones (*soyprx03*, *soyprx05*, *soyprx06*, *soyprx11*, *soyprx12*, and *soyprx14*) were chosen for further characterization since the 1.3 kb insert size matched the expected peroxidase transcript size. Sequence analysis of the six clones showed that they contained identical cDNA transcripts encoding a peroxidase and that each resulted

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from an independent cloning event since the junction between the cloning vector and the transcript was different in all cases.

Since it was not clear that the entire 5' end of the cDNA transcript was complete in any of the cDNA clones isolated, the structural gene corresponding to the seed coat peroxidase was isolated from a Harosoy 63 genomic library. A partial *Bam*HI  
5 digest of genomic DNA was used to construct the library and more than 10<sup>6</sup> plaque forming units were screened using the cDNA probe. A positive clone, G25-2-1-1-1, containing a 17 kb insert was identified and a 4.7 kb region encoding the peroxidase was sequenced SEQ ID NO:2. This region includes 1532 nucleotides of the 5' region  
10 of the peroxidase gene.

The genomic sequence matched the cDNA sequence except for three introns encoded within the gene. The genomic sequence also revealed two additional translation start codons, beginning one bp and 10 bp upstream from the 5' end of the  
15 longest cDNA transcript isolated. Figure 1 shows the deduced cDNA sequence. The open reading frame of 1056 bp encodes a 352 amino acid protein of 38,106 Da. A heme-binding domain, a peroxidase active site signature sequence, and seven potential N-glycosylation sites were identified from the deduced amino acid sequence. The first  
20 26 amino acid residues conform to a membrane spanning domain. Cleavage of this putative signal sequence releases a mature protein of 326 residues with a mass of 35,377 Da and an estimated pI of 4.4.



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Relevant features of the genomic fragment (Figure 2) include four exons at bp 192-411 (exon 1; 1533-1751 of SEQ ID NO:2), 1042-1233 (exon 2; 2383-2574 of SEQ ID NO:2), 2263-2429 (exon 3; 4033-4516 of SEQ ID NO:2) and 2692-3174 (exon 4; 1752-2382 of SEQ ID NO:2) and three introns at bp 412-1041 (intron 1; 1752-2382 of SEQ ID NO:2), 1234-2263 (intron 2; 2575-3604 of SEQ ID NO:2) and 2430-2691 (intron 3; 3770-4032 of SEQ ID NO:2). The 1532 bp regulatory region of the genomic DNA include a TATA box centred on bp 1487 and a cap signal 32 bp downstream centred at bp 1520 of SEQ ID NO:2. Also noted within the genomic sequence are three polyadenylation signals centred on bp 4520, 4598, 4700 and a polyadenylation site at bp 4700 of SEQ ID NO:2.

10

Figure 3 illustrates the relationship between the soybean seed coat peroxidase and other selected plant peroxidases. The soybean sequence is most closely related to four peroxidase cDNAs isolated from alfalfa, (see Figure 3) sharing from 65 to 67% identity at the amino acid level with the alfalfa proteins (X90693, X90694, X90692, el-Turk et al 1996; L36156, Abrahams et al 1994). When compared with other plant peroxidases, soybean seed coat peroxidase exhibits from 60 to 65% identity with poplar (D30653 and D30652, Osakabe et al 1994) and flax (L0554, Omann and Tyson 1995); 50 to 60% identity with horseradish (M37156, Fujiyama et al. 1988), tobacco (D11396, Osakabe et al 1993), and cucumber (M91373, Rasmussen et al. 1992); and 49% identity with barley (L36093, Scott-Craig et al. 1994), wheat (X85228, Baga et al 1995) and tobacco (L02124, Diaz-De-Leon et al 1993) peroxidases.

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A comparison of the promoter region, 1-1532 of SEQ ID NO:2, indicates that there are no similar sequences present within the GENBANK database.

**Example 2:           *DNA Blot Analysis Using the Seed Coat Peroxidase cDNA Probe Reveals Restriction Fragment Length Polymorphisms Between EpEp and epep Genotypes***

Genomic DNA biots of OX347 (*EpEp*) and OX312 (*epep*) plants were hybridized with <sup>32</sup>P-labelled cDNA to estimate the copy number of the seed coat peroxidase gene and to determine if this locus is polymorphic between the two genotypes. Figure 4 shows the hybridization patterns after digestion with *Bam*HI, *Xba*I, and *Sac*I. Restriction fragment length polymorphisms are clearly visible in the *Bam*HI and *Sac*I digestions. The *Bam*HI digestion produced a strongly hybridizing 17 kb fragment and a faint 3.4 kb fragment in the *EpEp* genotype. The 3.4 kb *Bam*HI fragment is visible in the *epep* genotype but the 17 kb fragment has been replaced by a signal at > 20 kb. The *Sac*I digestion resulted in detection of three fragments in *EpEp* and *epep* plants. At least two fragments were expected here since the cDNA sequence has a *Sac*I site within the open reading frame. However, the smallest and most strongly hybridizing of these fragments is 5.2 kb in *EpEp* plants and 4.9 kb in *epep* plants. Digestion with *Xba*I produced hybridizing fragments of ~14 kb and 7.8 kb for both genotypes, with the larger fragment showing a stronger signal.

**Example 3:** *A Deletion Mutation Occurs in the Recessive ep Locus*

The structural gene encoding the seed coat peroxidase is schematically illustrated in Figure 5. The 17 kb *Bam*HI fragment encompassing the gene includes 191 bp of sequence upstream from the translation start codon, three introns of 631 bp, 1030 bp, and 263 bp, and 13 kb of sequence downstream from the polyadenylation site. The arrangement of four exons and three introns and the placement of introns within the sequence is similar to that described for other plant peroxidases (Simon, 1992; Osakabe *et al.* 1995).

Primers were designed from the DNA sequence to compare *EpEp* and *epep* genotypes by PCR analysis. Figure 6 shows PCR amplification products from four different primer combinations using OX312 (*epep*) and OX347 (*EpEp*) genomic DNA as template. The primer annealing site for prx29+ begins 182 bp upstream from the ATG start codon; the remaining primer sites are shown in Figure 1. Amplification with primers prx2+ and prx6-, and with prx12+ and prx10- produced the expected products of 1.9 kb and 860 bp, respectively, regardless of the *Ep/ep* genotype of the template DNA. However, PCR amplification with primers prx9+ and prx10-, and with prx29+ and prx10- generated the expected products only when template DNA was from plants carrying the dominant *Ep* allele. When template DNA was from an *epep* genotype, no product was detected using primers prx9+ and prx10- and a smaller product was amplified with primers prx29+ and prx10-. The products resulting from amplification of OX312 or OX347 template DNA with primers prx29+ and prx10-

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were directly sequenced and compared. The polymorphism is due to an 87 bp deletion occurring within this DNA fragment in OX312 plants, as shown in Figure 5. This deletion begins nine bp upstream from the translation start codon and includes 78 bp of sequence at the 5' end of the open reading frame, including the prx9+ primer annealing site.

5

To test whether this deletion mutation cosegregates with the seed coat peroxidase phenotype, genomic DNA from an F<sub>2</sub> population segregating at the *Ep* locus was amplified using primers prx9+ and prx10- and F<sub>2</sub> seed was tested for seed coat peroxidase activity. Figure 7 shows the results from this analysis. Of the 30 F<sub>2</sub> individuals tested, all 23 that were high in seed coat peroxidase activity produced the expected 860 bp PCR amplification product. The remaining seven F<sub>2</sub>'s with low seed coat peroxidase activity produced no detectable PCR amplification products.

10

Finally, to determine if the OX312(*epep*) and OX347(*EpEp*) breeding lines are representative of soybean cultivars that differ in seed coat peroxidase activity, several cultivars were tested by PCK analysis using primer combinations targeted to the *Ep* locus. Figure 8 shows results from this analysis of six different soybean cultivars, three each of the homozygous dominant *EpEp* and recessive *epep* genotypes. As observed with OX312 and OX347, amplification products of the expected size were produced with primers prx12+ and prx10- regardless of the genotype, whereas *epep* genotypes yielded no product with primers prx9+ and prx10- or a smaller fragment with primers prx29+ and prx10-.

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#### Example 4 Developmental Pattern of Expression of the *Ep* gene

The seed coat peroxidase mRNA levels were determined by hybridizing RNA gel blots with radio labelled cDNA probe. The figure illustrates the transcript abundance in various tissues of *epep* and *EpEp* plants. The mRNA accumulated to high levels in seed coat tissues of *EpEp* plants, especially in the later stages development when whole seed fresh weight exceeded 50 mg. Low levels of transcript could also be detected in root tissues but not in the flower, embryo, pod or leaf. The transcript could also be detected in seed coat and root tissues *epep* plants but in drastically reduced amounts compared to the *EpEp* genotype. The reduced amounts of peroxidase mRNA present in seed coats of *epep* plants indicates that the transcriptional process and/or the stability of the resulting mRNA is severely affected. The *Ep* gene has a TATA box and a 5' cap signal beginning 47 bp and 15 bp, respectively, upstream from the translation start codon. The 87 bp deletion in the *ep* allele extends into the 5' cap signal and therefore could interfere with transcript processing. Regardless, any resulting transcript will not be properly translated since the AUG initiation codon and the entire amino-terminal signal sequence is deleted from the *ep* allele. Not wishing to be bound by theory, the lack of peroxidase accumulation in seed coats of *epep* plants appears to be due to at least two factors, greatly reduced transcript levels and ineffective translation, resulting from mutation of the structural gene encoding the enzyme. In summary, the results indicate that the *Ep* gene regulatory elements can drive high level expression in a tightly coordinated, tissue and developmentally specific manner.

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All scientific publications and patent documents are incorporated herein by reference.

The present invention has been described with regard to preferred  
5 embodiments. However, it will be obvious to persons skilled in the art that a number  
of variations and modifications can be made without departing from the scope of the  
invention as described in the following claims.

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## SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Seed Coat IMA Regulatory Region and  
Peroxidase

15 (iii) NUMBER OF SEQUENCES: 2

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: Patent In Release 01.0, Version 01.10 (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

25

## (1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1244 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
30 (D) TOPOLOGY: linear

(1v) ANTI-SENSE: NO

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(B) LOCATION: 1..1056

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(B) LOCATION: 1..77

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(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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TTC GAT GCT TCT TTC ACC GAT CCC CGA ATC GGG GCC AGT CTC ATG AGG 192

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	Phe	Asp	Ala	Ser	Phe	Thr	Asp	Pro	Arg	Ile	Gly	Ala	Ser	Leu	Met	Arg	
	50						55									60	
	CTT CAT TTT CAT GAT TGC TTT GTT CAA GGT TGT GAT GGA TCA GTT TTG																240
	Leu His Phe His Asp Cys Phe Val Gln Gly Cys Asp Gly Ser Val Leu																
5	65					70					75					80	
	CTG AAC AAC ACT GAT ACA ATA GAA AGC GAG CAA GAT GCA CTT CCA AAT																288
	Leu Asn Asn Thr Asp Thr Ile Glu Ser Glu Gln Asp Ala Leu Pro Asn																
						85					90					95	
10																	
	ATC AAC TCA ATA AGA GGA TTT GAC GTT GTC AAT GAC ATC AAG ACA GCG																336
	Ile Asn Ser Ile Arg Gly Leu Asp Val Val Asn Asp Ile Lys Thr Ala																
						100					105					110	
	GTG GAA AAT AGT TGT CCA GAC ACA GTT TCT TGT JCT GAT ATT CTT ACT																384
15	Val Glu Asn Ser Cys Pro Asp Thr Val Ser Cys Ala Asp Ile Leu Ala																
						115					120					125	
	ATT GCA GCT GAA ATA GCT TCT GTT CTG GGA GGA GGT CCA GGA TGG CCA																432
20	Ile Ala Ala Glu Ile Ala Ser Val Leu Gly Gly Gly Pro Gly Trp Pro																
						130					135					140	
	GTT CCA TTA GGA AGA AGG GAC AGC TTA ACA GCA AAC GGA ACC CTT GCA																480
	Val Pro Leu Gly Arg Arg Asp Ser Leu Thr Ala Asn Arg Thr Leu Ala																
25	145					150					155					160	
	AAT CAA AAC CTT CCA GCA CCT TTC TTC AAC CTC ACT CAA CTT AAA GCT																528
	Asn Gln Asn Leu Pro Ala Pro Phe Phe Asn Leu Thr Gln Leu Lys Ala																
						165					170					175	
30																	

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	TCC TTT GCT GTT CAA GGT CTC AAC ACC CTT GAT TTA GTT ACA CTC TCA	576
	Ser Phe Ala Val Gln Gly Leu Asn Thr Leu Asp Leu Val Thr Leu Ser	
	100 185 190	
5	GGT GGT CAT ACG TTT GGA AGA GCT CCG TGC AGT ACA TTC ATA AAC CGA	624
	Gly Gly His Thr Phe Gly Arg Ala Arg Cys Ser Thr Phe Ile Asn Arg	
	195 200 205	
10	TTA TAC AAC TTC AGC AAC ACT GGA AAC CCT GAT CCA ACT CTG AAC ACA	672
	Leu Tyr Asn Phe Ser Asn Thr Gly Asn Pro Asp Pro Thr Leu Asn Thr	
	210 215 220	
15	ACA TAC TTA GAA GTA TTG GGT GCA AGA TGC CCC CAG AAT GCA ACT GGG	720
	Thr Tyr Leu Glu Val Leu Arg Ala Arg Cys Pro Gln Asn Ala Thr Gly	
	225 230 235 240	
20	GAT AAC CTC ACC AAT TTG GAC CTG AGC ACA CCT GAT CCA TTT GAC AAC	768
	Asp Asn Leu Thr Asn Leu Asp Leu Ser Thr Pro Asp Gln Phe Asp Asn	
	245 250 255	
25	AGA TAC TAC TCC AAT CTT CTG CAG CTC AAT GGC TTA CTT CAG AGT GAC	816
	Arg Tyr Tyr Ser Asn Leu Leu Gln Leu Asn Gly Leu Leu Gln Ser Asp	
	260 265 270	
30	CAA GAA CTT TTC TCC ACT CCT GGT GCT GAT ACC ATT CCC ATT GTC AAT	864
	Gln Glu Leu Phe Ser Thr Pro Gly Ala Asp Thr Ile Pro Ile Val Asn	
	275 280 285	
30	ATC TTC ACG AGT AAC CAG AAT ACT TTC TTT TCC AAC TTT AGA GTT TCA	912
	Ser Phe Ser Ser Asn Gln Asn Thr Phe Phe Ser Asn Phe Arg Val Ser	
	290 295 300	

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ATG ATG AAA ATG GGT AAT ATT GGA GTG CTG ACT GCG GAT GAA GGA GAA 960  
 Met Il Lys Met Gly Asn Ile Gly Val Leu Thr Gly Asp Glu Gly Glu  
 305 310 315 320

ATT CGC TTG CAA TGT AAT TTT GTG AAT GGA GAC TCG TTT GGA TTA GCT 1008  
 5 Ile Arg Leu Gln Cys Asn Phe Val Asn Gly Asp Ser Phe Gly Leu Ala  
 325 330 335

AGT GTG GCG TCC AAA GAT GCT AAA CAA AAG CTT GTT GCT CAA TCT AAA 1056  
 Ser Val Ala Ser Lys Asp Ala Lys Gln Lys Leu Val Ala Gln Ser Lys  
 10 340 345 350

TAAACCAATA ATTAATGGGG ATGTGCATGC TAGCTAGCAT GTAAAGCCAA ATTACCTTGT 1116

AAACCTCTTT GCTAGCTATA TTGAATATA CCAGAGGAGT AGTGTGCATG TCAATTGGAT 1176  
 15

TTGCCATGT ACCTCTTGGG ATATTATGTA AEAATTATTT GAATCTCTTT AAGGTACTTA 1236

ATTAATCA 1244

20

## (2) INFORMATION FOR SEQ ID NO: 2:

## (1) SEQUENCE CHARACTERISTICS:

25

- (A) LENGTH: 4700 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

- (11) MOLECULE TYPE: DNA (genomic)

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## (ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION:1..1532

5

## (ix) FEATURE:

(A) NAME/KEY: sig\_peptide

(B) LOCATION:1533..1609

## (ix) FEATURE:

10

(A) NAME/KEY: exon

(B) LOCATION:1533..1751

## (ix) FEATURE:

15

(A) NAME/KEY: exon

(B) LOCATION:2383..2574

## (ix) FEATURE:

20

(A) NAME/KEY: exon

(B) LOCATION:3605..3769

## (ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:4033..4516

25

## (ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:1752..1782

## (ix) FEATURE:

30

(A) NAME/KEY: intron



- 47 -

(B) LOCATION:2575..3604

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:3770..4032

5

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1533..1751

10

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:2303..2574

(ix) FEATURE:

15

(A) NAME/KEY: CDS

(B) LOCATION:3605..3769

(ix) FEATURE:

20

(A) NAME/KEY: CDS

(B) LOCATION:4033..4516

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

25 TAGATAAAAA AATGGATAT AATTTTCCTC AGATGTTGTT TATAGTGT TTITAATCAG 60

AATTAATAAT CCTCTTAAT TATGACAA ATTTTTTTG GTGAATATTA TGTACATAAT 120

TATTAATAC AATTTTTAT TGTACATGA AGTGAATCTT CAATTTAAT ATTGGAGAAC 180

30

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	AGTACGAAAA CATAAAAAA CTGTTATTAG AAAAAAAA TATATGAAA AGGTTAGCTA	240
	CATATATTAG CTAAATTAGT TGTCTAATT GGCTATATAA ACCCTATGCT ACTCTTTGTA	300
5	ATCTCACTT TTTCATTAA ATACATTCT ACTTTTAAG TTCTATATT TCTCTAATT	360
	TTCTTGATA AACCATGAA TTAAACATG TATATCAGG ATACCAOCCA CTTTGAAAGC	420
	CATGTATGOC TAGTATGOC AGCCAAAAT TGCCTGGT CAAGCAAAGC AAGTGTATT	480
10	ATAGATGTGA CTTTGTGA GGAATCATG CCAATGGTAC TGATTGTGA ACTGAGAAAA	540
	CTAATTGGA GAATTGAAT TATGATCAT AAATACTCT CTCTGACTA CTTCTGCCC	600
	TCAAATTTGT ACCATCATTA TTCCCAAAA ATTGATTAC AATGCACTAA TTAATGAATG	660
15	TTCTTACAT TATCATATTA TCATATCTGA CATTTGTTT TTACTTTTA TAATAATTAT	720
	TTAAAAAGT CATACATGA AATAATTTT TAAATTTTA CATTAAAT TTACAGTAA	780
20	AAATCATGA AAATTAACT TTATTTTCC AATCATCAT TTAATCAAAT CCCAAACAA	840
	TGATTTT TTCAAATGA ATTTTATG AACATTAAA TTAGCCTAA TTAATCTGG	900
	TTATGTTTC AATTTCCAA AACCTAATC AATCTTAG CAATACATA CATAGATCTA	960
25	ATTTAACCT TATCTTAGC CAGAGATAT AAGATTAA CATCTAGTT TAAACATTA	1020
	CTTTGTTTT TGTGTAAAA AACATAACA TTTCCTAAT TTGTAGAGT GAGTGTCTC	1080
30	AACCATATTA ACGAGATT TATTGTTAT TCAATTCAT GAATCTAGTA AATAATTTT	1140

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5  
 10  
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 25  
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GGTCTTCAGT TTTCATTTT CATTACACA TTATGTAAA ATATCAAGT TTTCTGAAAT 1200  
 TTGTTGCTTG TGTGCTCCA CCACATTAA GAGATTATG AAATTAATTT TCAAGAAGAT 1260  
 AATGATTCTT ACTCTTGCTG GCGCTACCAT AGTACAATA ATCCACTCAT AAATCAACAA 1320  
 GTGTTGCTCA TAGGCAATTG GGCATCATAT CATAACAAT ACGTACGTGA TATTATCTAG 1380  
 TGTCTCTCAG TTACTTTAT GAGAAATAT TTTCTTTAA AAAAGTTAA TTAATAAAA 1440  
 CATTTCGAT ACCGTGATTT ACAAGAAATC GCGCGAATTC ATCTCTATAA ATAAAAGGAT 1500  
 CTATATGAGA GGTAAAATCA TATTAATCTA AA ATG GGT TCC ATG CGT CTA TTA 1553  
 Met Gly Ser Met Arg Leu Leu  
 355  
 GTA GTG GCA TTG TTG TOT GCA TTT GCT ATG CAT GCA GGT TTT TCA GTC 1601  
 Val Val Ala Leu Leu Cys Ala Phe Ala Met His Ala Gly Phe Ser Val  
 360 365 370 375  
 TCT TAT GCT CAG CTT ACT CCT ACG TTC TAC AGA GAA ACA TOT CCA AAT 1649  
 Ser Tyr Ala Gln Leu Thr Pro Thr Phe Tyr Arg Glu Thr Cys Pro Asn  
 380 385 390  
 CTG TTC CCT ATT GTG TTT GGA GGA ATC TTC GAT GCT TCT TTC ACC GAT 1697  
 Leu Phe Pro Ile Val Phe Gly Val Ile Phe Asp Ala Ser Phe Thr Asp  
 395 400 405  
 CCC CGA ATC GGG GCG AGT CTC ATG AGG CTT CAT TTT CAT GAT TGC TTT 1745  
 Pro Arg Ile Gly Ala Ser Leu Met Arg Leu His Phe His Asp Cys Phe  
 410 415 420

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GTT CAA GTACGTACTT TTTTTTTC TTCCAAAATG CCTGCAATAT TTAACAAGAT 1861  
 Val Gln  
 425

5 TGCTTTGTTT ACCTAGAAAA ATGTGTTTTT TTCAACGATC TTACGTACGT TTGTTTGTTT 1861  
 TGAAAAATAA ATCAGAAGA GATCAAGAAA ATAGCTAGAA AGAAAGCAAC GTTTTTTTAA 1921  
 AAGGTATTTA GTGTGAGAAA AATATTAATA CTGAAGAGAA AGAAATTAAT TAAGCTTTTC 1981

10 TTGAATGATA TTACATGTC TTATTAACCT AAGTCACCT TTTTCTTTA AGTTGTGCTT 2041  
 GAAGAAAAAA GATGCTTTTC AGTTTAAGTT TGATTAATGC TAATTATATT TTTAATTAAT 2101  
 TAATTAATAC TATATATCTA TTTACCAAT TAATTATTAC TATATTCAT GATGACAACA 2161

15 GACAAGTATT CTAAGAGGT ATCGGTAGAT GATTAATTTT TTTATAAAAA AATCTTTTGC 2221  
 GTGTATAGAT ATTCTTTTAT AATTGGTGCA GAAACTGTA ATGCTAATTG CAATTAATCT 2281

20 TACATTGATT AACTAATAGC TATAATCAAT ATTTAGGTTA GGTATAGAG ACAAATCAAG 2341  
 TGATCTGAAC AAATTAAGTT GTTATATTTG CATTGTGACA G GGT TGT GAT GCA 2394  
 Gly Cys Asp Gly  
 1

25 TCA GTT TTG CTG AAC AAC ACT GAT ACA ATA GAA AGC GAG CAA GAT GCA 2442  
 Ser Val Leu Leu Asn Asn Thr Asp Thr Ile Glu Ser Glu Gln Asp Ala  
 5 10 15 20

30 CTT CCA AAT ATC AAC TCA ATA AGA GGA TTG GAC GTT GTC AAT GAC ATC 2490

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Leu Pro Asn Ile Asn Ser Ile Arg Gly Leu Asp Val Val Asn Asp Ile

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AAG ACA GCG GTG GAA AAT AGT TGT CCA GAC ACA GTT TCT TGT GCT GAT 2538

Lys Thr Ala Val Glu Asn Ser Cys Pro Asp Thr Val Ser Cys Ala Asp

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ATT CTT GCT ATT GCA GCT GAA ATA GCT TCT GTT CTG GTAATTAATA 2584

Ile Leu Ala Ile Ala Ala Glu Ile Ala Ser Val Leu

55

60

10

ACTCCTAATT AATCCCTAC CATTAAGAAG TTGCATGATT GGATTCAAAA TTCTATGOTA 2644

TTGGGGTTCT GATATAAATT TGTAAATAAA TTGCACTAAA AAAAATTATC ATATACTTTT 2704

15 AATAAAAAAA ATTTATCTAA TTTAATTAAT TATTAAGACT ATTTTAAAA TTCAATCCTA 2764

ACTCTTTTTT AATCGAGCA TGTAACTGG CACCCACCGT ATATCGTTGG AAGATGCTAT 2824

AAAACCATTT AATTAATGGA TGTAACTGG CAAACATTT AATCAAAAT ACTCTTAATT 2884

20

GTGATTAGTA ATCATGTTGG GGCAGTTCAC GTTGTGTATA ATTAATTGGA CTTAATCAGA 2944

TAAAAAACA AATGACUCA AGCGGTTGG TATAGATATC ACTGGCCTGT AGTATATGTT 3004

25 GTTTTCACG TTAAATGAA AGCTAGCTAC TATATTATAT TTAGTCTTTT TTTTCTEAA 3064

ACCCATTEAA GTGATTAT TGAATGTGAA ACATGTTTCC ACACACAGGC TTAGAAACTC 3124

CTGCAACTA ACATCTCCA AATTGACTA TTTATTTATG AAGATTAATC ATCTATGATG 3184

30

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TTCAACTCTA TTATATATAT GTATCATGCG AGTATTAAGA ATTATAATAG TCAAATATAG 3244  
 AAGTATATCG GGTAAATGTA GTTCATGTG CGACCTGTTT CGTGTAATAT GCTTATTCTA 3304  
 TATAGCTTTT TTTATTGGAA AATAACGATG AACTAAAAAC GAAAGGGTAT CATATAGTTT 3364  
 5 GACTTTTATG TTAGAGAGAG ACATCTTAAT TTGOTCAEAT GTTAAATAAT TAATTACAAT 3424  
 GCATACACAA ATATTATGCG CATATCTAAA AATGATAAAA ATATCATAGG TAACTCAAC 3484  
 10 TATATGATAT CCCATAACA GAAATTGTAC TTTCTTCAG GCAATGAAC TAACTTTCT 3544  
 GTTCTCTAAA AACAAACATC CACTTAAAGT GGTTCACAT ATTTATGTAA TAATTTACAG 3604  
 GGA GGA GGT CCA GGA TGG CCA GTT CCA TTA GGA AGA AGG GAC AGC TTA 3652  
 15 Gly Gly Gly Pro Gly Trp Pro Val Pro Leu Gly Arg Arg Asp Ser Leu  
 1 5 10 15  
 ACA GCA AAC CGA ACC CTT GCA AAT CAA AAC CTT CCA GCA CCT TTC TTC 3700  
 Thr Ala Asn Arg Thr Leu Ala Asn Gln Asn Leu Pro Ala Pro Phe Phe  
 20 20 25 30  
 AAC CTC ACT CAA CTT AAA GCT TCC TTT GCT GTT CAA GGT CTC AAC ACC 3748  
 Asn Leu Thr Gln Leu Lys Ala Ser Phe Ala Val Gln Gly Leu Asn Thr  
 35 40 45  
 25 CTT GAT TTA GTT ACA CTC TCA GGTATACATA ATCAATTTT TATTTGCTAT 3799  
 Leu Asp Leu Val Thr Leu Ser  
 50 55  
 30 TAGCTAGCAA TAAAAAGTCT CTGATACAGA CATATTAGA TAAATTAAT TCTCCATAAA 3859

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	CATTATAAT AAAATTATCA ATTATGTAC TAAAAATTA TGGATTGAAG CTCTTTTCAT	3919
	CCAACTTTTA CTAAAGTTAA GGTGCATATA ATATAAAATA AACTATCTCT TGTTCCTAT	3979
5	AAAAAGATTG AAGATAAGTT AAGTCTACT TATAAATCAT TAATATATGT ATA GGT	4035
		Gly
		1
10	GGT CAT ACC TTT GGA AGA OCT GGG TGC AGT ACA TTC ATA AAC CGA TTA	4083
	Gly His Thr Phe Gly Arg Ala Arg Cys Ser Thr Phe Ile Asn Arg Leu	
	5 10 15	
15	TAC AAC TTC AGC AAC ACT GGA AAC CCT GAT CCA ACT CTG AAC ACA ACA	4131
	Tyr Asn Phe Ser Asn Thr Gly Asn Pro Asp Pro Thr Leu Asn Thr Thr	
	20 25 30	
20	TAC TTA GAA GTA TTG CGT GCA AGA TGC CCC CAG AAT GCA ACT GGG GAT	4179
	Tyr Leu Glu Val Leu Arg Ala Arg Cys Pro Gln Asn Ala Thr Gly Asp	
	35 40 45	
25	AAC CTC ACC AAT TTG GAC CTG AGC ACA CCT GAT CAA TTT GAC AAC AGA	4227
	Asn Leu Thr Asn Leu Asp Leu Ser Thr Pro Asp Gln Phe Asp Asn Arg	
	50 55 60 65	
30	TAC TAC TCC AAT CTT CTG CAG CTC AAT GGC TTA CTT CAG AGT GAC CAA	4275
	Tyr Tyr Ser Asn Leu Leu Gln Leu Asn Gly Leu Leu Gln Ser Asp Gln	
	70 75 80	
35	GAA CTT TTC TCC ACT CCT GGT GCT GAT ALC ATT CCC ATT GTC AAT AGC	4323
	Glu Leu Phe Ser Thr Pro Gly Ala Asp Thr Ile Pro Ile Val Asn Ser	
	85 90 95	

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	TTC AGC AGT AAC CAG AAT ACT TTC TTT TCC AAC TTT AGA GTT TCA ATG	4371
	Phe Ser Ser Asn Gln Asn Thr Phe Phe Ser Asn Phe Arg Val Ser Met	
	100 105 110	
5	ATA AAA ATG GGT AAT ATT GGA GTG CTG ACT GGG GAT GAA GGA GAA ATT	4419
	Ile Lys Met Gly Asn Ile Gly Val Leu Thr Gly Asp Glu Gly Glu Ile	
	115 120 125	
10	CGC TTG CAA TGT AAT TTT GTG AAT GGA GAC TCG TTT GGA TTA GCT AGT	4467
	Arg Leu Gln Cys Asn Phe Val Asn Gly Asp Ser Phe Gly Leu Ala Ser	
	130 135 140 145	
15	GTG GCG TCC AAA GAT GCT AAA CAA AAG CTT GTT GCT CAA TCT AAA TAA	4515
	Val Ala Ser Lys Asp Ala Lys Gln Lys Leu Val Ala Gln Ser Lys	
	150 155 160	
	ACCAATAATT AATGGGGATG TGCATGCTAG CTAGCATGTA AAGCCAAATT AGTTGTAAA	4575
	CCTCTTTGCT AGCTATATTG AAATAAACCA AAGGAGTAGT GTGCATGTCA ATTGATTTT	4635
20	GCCATGTACC TCTTGGPATA TTATGTAAEA ATTATTGAA TCTCTTTAAG GTACTTAATT	4695
	AATCA	4700



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**THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE  
PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:**

1. An isolated DNA molecule comprising the nucleotide sequence of SEQ ID NO:1.
2. An isolated DNA molecule comprising at least 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2
3. The isolated DNA molecule comprising a nucleotide sequence substantially homologous to nucleotides 1533-4700 of SEQ ID NO:2.
4. The isolated DNA molecule of claim 3 comprising a nucleotide sequence substantially homologous to that of nucleotides 1-4700 of SEQ ID NO:2.
5. The isolated DNA molecule of claim 3 comprising nucleotides 1533-4700 of SEQ ID NO:2.
6. The isolated DNA molecule of claim 4 comprising the nucleotide sequence of SEQ ID NO:2.
7. The isolated DNA molecule of claim 2 comprising a nucleotide sequence substantially homologous to that of 1-1532 of SEQ ID NO:2.
8. The isolated DNA molecule of claim 7, comprising the nucleotide sequence of nucleotides 1-1532 of SEQ ID NO:2.
9. An isolated DNA molecule of claim 3 comprising at least 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ ID NO:2.

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10. An isolated DNA molecule of claim 9 comprising the nucleotide sequence of 412-1041 of SEQ ID NO:2.
11. An isolated DNA molecule of claim 3 comprising at least 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ ID NO:2.
12. An isolated DNA molecule of claim 11 comprising the nucleotide sequence of 1234-2263 of SEQ ID NO:2.
13. An isolated DNA molecule of claim 3 comprising at least 22 contiguous nucleotides selected from nucleotides 2430-2691 of SEQ ID NO:2.
14. An isolated DNA molecule of claim 13 comprising the nucleotide sequence of 2430-2691 of SEQ ID NO:2.
15. A vector which comprises the DNA molecule of claim 1.
16. A vector which comprises the DNA molecule of claim 2.
17. A vector which comprises the DNA molecule of claim 3.
18. The vector of claim 16 which comprises a heterologous gene of interest under control of the DNA molecule.
19. A host cell capable of expressing the DNA molecule within the vector of claim 15.
20. A host cell capable of expressing the DNA molecule within the vector of claim 16.

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21. A host cell capable of expressing the DNA molecule within the vector of claim 17.
22. A host cell capable of expressing the DNA molecule within the vector of claim 18.
23. A transgenic plant comprising the vector of claim 15.
24. A transgenic plant comprising the vector of claim 16.
25. A transgenic plant comprising the vector of claim 17.
26. A transgenic plant comprising the vector of claim 18.
27. A method for the production of soybean seed coat peroxidase in a host cell comprising:
  - i) transforming the host cell with a vector comprising an isolated DNA molecule selected from the group consisting of SEQ ID NO:1, and SEQ ID NO:2; and
  - ii) culturing the host cell under conditions to allow expression of the soybean seed coat peroxidase.
28. A process for producing a heterologous gene of interest comprising propagating a transformed plant with the vector of claim 16.
29. The process of claim 28 wherein the heterologous gene of interest is produced within seed coat cells.

FIGURE 1

	ATGGGTTCCATGCGTCTATT	20
	<u>M G S M R L L</u>	
	----- prx9+ ----->	
AGTAGTGGCATTGTTGTGTGCATTGCTATGCATGCAGGTTTTTCAGTCTCTTATGCTCA	80	
<u>V V A L L C A P A M H A G F S V S Y A Q</u>	1	
signal sequence		
GCTTACTCCTACGTTCTACAGAGAAACATGTCCAAATCTGTTCCCTATTGTGTTTGGAGT	140	
<u>L T P T F Y R E T C P N L P P I V F G V</u>	21	
	----- prx12+ ----->	
AATCTTCGATGCTTCTTTCACOGATCCCCGAATCGGGGCCAGTCTCATGAGGCTTCATTT	200	
<u>I F D A S F T D P R I Q A S L M R L H F</u>	41	
active site		
	I	
TCATGATTGCTTTGTTCAAG GTTGTGATGGATCAGTTTTGCTGAACAACTGATACAAT	260	
<u>H D C F V Q G C D G S V L L N N T D T I</u>	61	
	----- prx10- ----	
	----- prx2+ ----->	
AGAAAGCGAGCAAGATGCACTTCCAAATATCAACTCAATAAGAGGATTGGACGTTGTCAA	320	
<u>E S E Q D A L P M I N S I R G L D V V N</u>	81	
TGACATCAAGACAGCGGTGGAAAATAGTTGTCCAGACACAGTTTCTTGTGCTGATATTCT	390	
<u>D I K T A V E N S C P D T V S C A D I L</u>	101	
	II	
TGCTATTGCAGCTGAAATAGCTTCTGTTCTG GGAGGAGGTCCAGGATGGCCAGTTCCATT	440	
<u>A I A A E I A S V L G G G P G N P V P L</u>	121	
AGGAAGAAGGGACAGCTTAPCAGCAAAACCGAACCTTGCAAATCAALACCTTCCAGCACC	500	
<u>G R R D S L T A N R T L A N Q J L P A P</u>	141	
TTTCTTCAACCTCACTCAACTTAAGCTTCCCTTTGCTGTTCAAGGTCTCAACACCCCTTGA	560	
<u>F P N L T Q L K A S P A V Q G L N T L D</u>	161	
	III	
TTTAGTTACACTCTCAG GTGGTCATACGTTTGGAGAGCTCGGTGCAGTACATTTCATAAA	620	
<u>L V T L S G G H T F G R A R C S T F I N</u>	181	
heme-binding domain		
CGGATTATACAACCTTCACCAACACTGGAAACCCCTGATCCAACCTCTGAACACAACATACTT	680	
<u>R L Y N F S N T G M P D P T L N T T Y L</u>	201	
AGAAGTATTGCGTGCAAGATGCCCGAGAATGCAACTGGGGATAACCTCACCAATTGGA	740	
<u>E V L R A R C P Q N A T G D N L T N L D</u>	221	
CCTGAGCACACCTGATCAATTTGACAAACAGATACTACTCCAATCTTCTGCAGCTCAATGG	900	
<u>L S T P D Q F D M R Y Y S N J L Q L N G</u>	241	
CTTACTTCAGAGTGACCAAGAACCTTTTCTCCACTCCTGGTGCTGATACCATTCCTTGT	860	

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L L Q S D Q E L P S T P G A D T I P I V 261  
 <----- PER6- ----->  
 CAATAGCTTCAGCAGTAACCGAATACTTTCTTTTCCAACTTTAGAGTTTCAATGATAAA 920  
 N S P S S N Q N T P P S N P R V S M I K 281  
 AATGGGTAAATATTGGAGTGCTGACTGGGGATGAAGGAGAAATTCGCTTGCAATGTAATTT 980  
 M G N I G V L T G D E G E I R L Q C N F 301  
 TGTGAATGAGACTCGTTTGGATTAGCTAGTGTGGCGTCCAAAGATGCTAAACAAAAGCT 1040  
 V N G D S P G L A S V A S K D A K Q K L 321  
 TTTTGCTCAATCTAAATAAACCAATAATTAATGGGATGTGATGCTAGCTAGCATGTAA 1100  
 V A Q S K \* 326  
 AGGCAAATTAGGTTGTAAACCTCTTTGCTAGCTATATTGAAATAAACCAAAGGAGTACTG 1160  
 TGCATGTCAATTGGATTTTGCCATGTACCTCTTGGAAATATTATGTAATAATTATTGGAAT 1220  
 CTCTTTAAGGTACTTAATTAATC (A) B

Gowling, Strathy & Henderson

FIG. 2

10 20 30 40 50 60

1 GCATCATATCTATAACAAATACGTACGTGATATTATCTAGTGTCTCTCAGTTTACTTTTATG  
61 AGAATATTAATTTTCTTTAAAGAAAGTTAATTAAATAAAACATTTGGGATACCGTGAATTA  
121 CAGAAATCCGGCCGAATTCATCTCTATRAATTAAGAGATCTATATGAGAGGTTAATCAT  
181 ATTAACCAAAATCGGTTCATCTCTATTAATAGTATGGCATTGTTCTTTGCAATTTGCTA  
241 TCAATGAGGTTTTCAGTCTCTTATGCTCAGCTTACTCTACGTTTACAGAGACAT  
301 TCCAAATCTGTTCCCTATTGTGTTGGATTAATCTTUGATGCTTCTTTTACCCGATCCCC  
361 GAATCGGGGCCAGTCTCATGAGGCTTCATTTCAATGATTGCTTTGTTCAAGTACGTTACTT  
421 TTTTCTTTCTTCCAAATGCCCCGCAATTTTACAAGATTGCTTTGTTTACATAGAAAA  
481 AGTGTCTTTTCTTCAACCATCTTTCGTACGTTTGTGCTTTGAAATTAATAATCAGATAGA  
541 GATTAACAAATAGCTAGAAAGAAAGCAACGTTTCTTAAAGGTTTGTAGTGTGAGAAA  
601 AATATTAAGACTGAGAGAGAAAGAAATTAATAAGCTTTCTGTGATGATGATTTACATGTC  
661 TTATTAAGTTAAATCAAGCTTTTCTTTTAAGTCTTCTGAGAGAAAGATGCTTTCTC  
721 AGTGTAGTTTGTATTAATGCTAATTATATTTTAAATTAATTAATTAATTAATTAATTA  
781 TTTACATATTAATTTATTTATTTATTTTCAATGATGACACAGACAACTATTTCAAGAGGT  
841 ATCGATATATGATTAATTTTATTAATAAAATCTTTTGGGTGTATAGATATTTCTTTTAT  
901 AATGCTGTCAGAAATCTGTATGCTAATTGCAATTAATCTTACATTGATTAATTAATTAAT  
961 TATAATTAATTTAAGTTAGGTATAGGAGCAAAATCAATGATCTGAAACAAATTAAGTT  
1021 GTTATAATTTGCAATTTGACAGGCTTGTGATGGAATCAATTTGCTTAAGATCAATGATACA  
1081 ATAGTAAGGAGAGAGATGCACTTCAATATCACTCAATCAAGAGGATGAGGTTGCT  
1141 AATGACATCAATACAGCGGTGAGAAATCTTGTCTCAGACACAGTTCTCTGCTGATATT  
1201 CTGCTATCTGCACTGTAATAGCTCTGTCTGTGATTAATTAATTAATTAATTAATTAAT  
1261 AAGCAATTAAGAGTTGATGATGCAATTTCAAAATCTGATGATTTGAGGTTGATATAA  
1321 AATTTTAATTAATTTGCACTAAAAAAATATCATATATCTTTTAATAAAAAAATTTATC  
1381 TAAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTA  
1441 GCATGTAAGCTGGCACCACCGTATATCTTTGAGAGATGCTATATAATCAATTAATTAAT  
1501 GATGTAATCAATCAAAACATTTAATTAATTAATTAATTAATTAATTAATTAATTAATTAAT  
1561 TCGGCAAGTTAGCTTGTGATATAATTAATTAATTAATTAATTAATTAATTAATTAATTAAT  
1621 GTAGGCTGCTGGATAGATATCACTGGCTGTAGATTAATTAATTAATTAATTAATTAATTAAT  
1681 AAAAGCAGCTACTATATATATTTAGTCTTTTTTTTTTTCTTAAAGCAATTAAGCTGAT  
1741 TATTAATTTGAAACATCTTCCACACACAGGCTTAAGATCTTCCCAAGTAAATCTCTC  
1801 CAAATTTGATATTTTGAAGATAATTCATCTATGATGTCAGCTCTATTATATA  
1861 TATGATATATCGGAGTGAAGATTAATAGTCAAAATATAAATATATCGGATATAT  
1921 GTAGTTGCAATGTGCGACCTTTTCTGTGTAATAATTAATTAATTAATTAATTAATTAAT  
1981 GAAATTAAGCAATCACTAAAAACCAAGGATATCATATAGTTTGACTTTATATTTAGAGA  
2041 CAGACATCTTAATTTCTGTCATATGTTAATAATTAATTAATTAATTAATTAATTAATTAAT  
2101 TGACATATCTAATAAATGATATAATATCATAGGTATACCTCACTATATGATATCTGATA  
2161 ACAGAAATTTGATTTTCTTCAAGCAATGAATTAACCTTCTGTTTCTTAAAGCAAAAC  
2221 ATCCACTTAAGTGGTTCAACATATTAATGTAATAATTAACAGGAGGAGGCTCAGGATG  
2281 GCTAGTTCTATTAGGAAGAGGAGCACTTAACAGCAACCGTACCGCTTCAAAATCAAAAT  
2341 CCTTCCAGCACTTTCTTCAACCTCACTCAACTTAAAGCTTCTTCTGCTGTTCAAGGCTCT  
2401 CAACACCTTTGATTTAGTTTACCTCTCAGGTATACATAATCAATTTTATTTGCTATTA  
2461 GCTAGCAATAAAAGTCTCTGATACAGACATATTAAGATAAATTAATTTCTGATATAAACA  
2521 TTTATAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAATTAAT  
2581 AACTTTTACTAAAGTTAAGTGCATATAATATAAATAAATTAATTAATTAATTAATTAATTA  
2641 AAAGATTGAATATAAGTTAAGTCTAATTAATAATTAATTAATTAATTAATTAATTAATTAAT  
2701 ACGTTTGGAAAGCTCGGTGAGTTCATTCAATAACCAATTAATTAATTAATTAATTAATTAAT  
2761 GGAAGCTGATCACTCTGAACACAACTAATTAAGATTAAGGCTGAGAGATGAGGCTG  
2821 CAGAAATCAATCTGATTAACCTCACTAATTAAGATTAAGGCTGAGAGATGAGGCTGAGGCTG  
2881 AACAGATATCTCTCAATCTTCTGAGCTCAATGCTTACTTAAGTGAAGCAAGGCTT  
2941 TTTCTCACTCTGCTGCTGATACCATTTCTCAATTTCTCAATTTCTCAATTTCTCAATTTCT  
3001 ACTTTCTTTTCACTTTAGAGTTTCAATTAATAAATAAGTAAATTAATTAATTAATTAATTAAT

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3061 GGGGATGAAGGAGAAAATTCGCTTGCAATGTAATTTTGTGAATGGAGACTCGTTTGGATTA  
3121 GCTAGTGTGGCGTCCAAAGATGCTAAACAAAAGCTTGTGCTCAATCTAAATAAACCAAT  
3181 AATTAAATGGGATGTGCATGCTAGCTAGCATGTAAAGGCAAATTAGGTTGTAAACCTCTT  
3241 TGCTAGCTATATTGAAATAAACCAAAGGAGTAGTGTGCATOTCAATTCGATTTTGCCATG  
3301 TACCTCTTGGAAATATTATGTAATAATTATTTGAATCTCTTAAGGTACTTAATTAATCA

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FIGURE 3A

L 316	-----ATGGGTTCCATTCGT-CTATTAGTAGTGGCAT-CTTG	36
U41657	-----	0
X90693	G----GCAAA-CAATGAACTCCCTTCGTGCTGTAGCAATAG-CTTTGTGC	14
X90694	GCTCTTCAAAACAATGAACTCC-----TTAGCAACTT-CTATGTGG	40
L36156	-----CTCC-----TTAGCAACTT-CTATCTGG	22
X90692	-----AATGCTTGGT-----CTAAGTGCAACAGCTTTTTTGTCTGTATGC	38
L78163	TGT-----GCATTT-GCTATGCATGCAGGTTTTTTCAGT---CTCTTATGC	77
U41657	-----	0
X90693	TGTATTCTG-----GTTGTGCTTGGAGGGTTACCTTCTCTTCAAAATGC	88
X90694	TGTG-TGTGCTTTTAGTTGTGCTTGGAGGACTACCTTTTTCTCAGATGC	90
L36156	TGTGTTGTGCTTTTAGTTGTGCTTGGAGGACTACCTTTTCTCAGATGC	72
X90692	TT-TT-TGCTAAT-----TGGAGGAGTACCTTTT---CAAATGC	75
L78163	TCAGCTTACTCCTACGTTCTACAGAGAAACATGTCCAAATCTGTTCCCTA	127
U41657	-----	0
X90693	GCAACTTGATCCATCCTTTTACAGGAAACCTTGTCCAAATGTTAGTTCCA	138
X90694	ACAACCTTAGTCCCACTTTTTACAGCAAAACGTGTCCAACTGTTAGTTCCA	140
L36156	ACAACCTTAGTCCCACTTTTTACAGCAAAACGTGTCCAACTGTTAGTTCCA	122
X90692	ACAACCTAGATCCTTCATTACAACTACATGTTCTAATCTTGATTCAA	125
L78163	TTGTGTTTGGAGTAATCTTCGATGCTTCTTTACCGATCCCGAATCGGG	177
U41657	-----	0
X90693	TTGTTCGTGAAGTCATAAGGAGTGTTCCTAAGAAAGATCCTCGTATGCTT	188
X90694	TTGTTAGCAATGTCTTAACAAACGTTTCTAAGACAGATCCTCGCATGCTT	190
L36156	TTGTTAGCAATGTCTTAACAAACGTTTCTAAGACAGATCCTCGCATGCTT	172
X90692	TCGTACGTGGTGTGCTCACAAATGTTTCACAATCTGATCCCAAGATGCTT	175
L78163	GCCAGTCTCATGAGGCTTCATTTTCATGATGCTTTCTTCAGGTTGTGA	227
U41657	-----TTTCTGATTGCTTTGTTTCAGGTTGTGA	29
X90693	GCTAGTCTTGTGAGGCTTCACCTTCATGACTGTTTTGTTCAAGGTTGTGA	238
X90694	GCTAGTCTTGTGAGGCTTCACCTTCATGACTGTTTTGTTCTGGGATGTGA	240
L36156	GCTAGTCTTGTGAGGCTTCACCTTCATGACTGTTTTGTTCTGGGATGTGA	222
X90692	GGTAGTCTCATGAGGCTACATTTTCATGACTGTTTTGTTCAAGGTTGTGA	225
L78163	TTGATCAGTTTTTCTGAACAACCTGATACAAATAGAAAGCCAGCAAGATG	277
U41657	TGGATCAGTTTTTACTGAACAACCTGATACAAATAGAAAGCCAGCAAGATG	79
X90693	TGCATCAGTTTTTACTGAACAACCTGATACCGTTGTGAGTGAACAAGATG	288
X90694	TGCCCTCAGTTTTTCTGAACAATACTGCTACAATCGTAAGCGAACAACAAG	290
L36156	TGCCCTCAGTTTTTCTGAACAATACTGCTACAATCGTAAGCGAACAACAAG	272
X90692	TGCCCTCAGTTTTTCTGAACGATACGGCTACAATAGTGAGCGAGCAAGTG	275
L78163	CACTTCCAAATATCAACTCAATAAGAGGATTGGACGTTGTCAATGACATC	327
U41657	CACTTCCAAATATCAACTCAATAAGAGGATTGGACGTTGTCAATGACATC	129
X90693	CTTTTCCAAACAGAAATCTCATTAAAGAGTTTGGATGTTGTGAATCAAATC	338
X90694	CTTTTCCAAATAACAACCTCTCTAAGAGGTTTGGATGTTGTGAATCAGATC	340
L36156	CTTTTCCAAATAACAACCTCTCTAAGAGGTTTGGATGTTGTGAATCAGATC	322
X90692	CACCACCAATAACAACCTCATAAGAGGTTTGGATGTTGAATAACCAGATC	325
L78163	AAGACAGCGGTGAAAAATAGTTGTCCAGACACAGTTTCTTGTGCTATAT	377

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U41657	AAGACAGCGGTGGAAAATAGTTGTCCAGACACAGTTTCTTTGTGCTGATAT	179
X90693	AAAACAGCTGTGGAAAAGGCTTGTCTTAACACAGTTTCTTGTGCTGATAT	189
X90694	AAGCTGGCTGTAGAAGTGCCCTGTCTTAACACAGTTTCTTGTGCTGATAT	190
L36156	AAGCTGTCTGTAGAAGTGCTTGTCTTAACACAGTTTCTTGTGCTGATAT	172
X90692	AAAACAGCGGTGGAAAATAGTTGTCTTAACACAGTTTCTTGTGCTGATAT	175
	*** **	
L78163	TCTTGCTATTGCAGCTGAATAAGCTTGTGTC-CTGGCAGGAGGTCCAGGA	426
U41657	TCTTGCTATTGCAGCTGAATAAGCTTGTGTC-CTGGCAGGAGGTCCAGGA	228
X90693	TCTTGCTATTGCAGCTGAATAAGCTTGTGTC-CTGGCAGGAGGTCCAGGA	437
X90694	TCTTGCTATTGCAGCTGAATAAGCTTGTGTC-CTGGCAGGAGGTCCAGGA	439
L36156	TCTTGCTATTGCAGCTGAATAAGCTTGTGTC-CTGGCAGGAGGTCCAGGA	418
X90692	TCTTGCTATTGCAGCTGAATAAGCTTGTGTC-CTGGCAGGAGGTCCAGGA	424
	***** **	
L78163	TGGCCAGTTCCATTAGGAAGAAGGACAGCTTAACAGCAAAACGAAACCT	476
U41657	TGGCCAGTTCCATTAGGAAGAAGGACAGCTTAACAGCAAAACGAAACCT	279
X90693	TGGAAAGTTCCATTAGGAAGAAGGACAGCTTAACAGCAAAACGAAACCT	487
X90694	TGGAAAGTTCCATTAGGAAGAAGGACAGCTTAACAGCAAAACGAAACCT	489
L36156	TGGAAAGTTCCATTAGGAAGAAGGACAGCTTAACAGCAAAACGAAACCT	468
X90692	TGGAAAGTTCCATTAGGAAGAAGGACAGCTTAACAGCAAAACGAAACCT	474
	*** **	
L78163	TGCAAAATCAAAAACCTTCCAGCACCCTTCTTCAA--CTTCA-CTCAACTTA	523
U41657	TGCAAAATCAAAAACCTTCCAGCACCCTTCTTCAA--CTTCA-CTCAACTTA	325
X90693	TGCAAAATCAAAAACCTTCCAGCACCCTTCTTCAA--CTTCA-CTCAACTTA	534
X90694	TGCAAAATCAAAAACCTTCCAGCACCCTTCTTCAA--CTTCA-CTCAACTTA	536
L36156	TGCAAAATCAAAAACCTTCCAGCACCCTTCTTCAA--CTTCA-CTCAACTTA	515
X90692	TGCAAAATCAAAAACCTTCCAGCACCCTTCTTCAA--CTTCA-CTCAACTTA	521
	*** **	
L78163	AAGCTTCCCTTG-CTGTTCAAGGTCTCAACACCCTTGATTTAGTTACACT	572
U41657	AAGCTTCCCTTG-CTGTTCAAGGTCTCAACACCCTTGATTTAGTTACACT	374
X90693	AAGCTTCCCTTG-CTGTTCAAGGTCTCAACACCCTTGATTTAGTTACACT	583
X90694	AAGCTTCCCTTG-CTGTTCAAGGTCTCAACACCCTTGATTTAGTTACACT	585
L36156	AA-CTGCATCTGACTGCTCAAGGCTCTTACTCTCTTCTAGTTGCCCT	564
X90692	AATCTAATCTTGA-TAATTAAAACCTCACTACTACTGATCTAGTTGCACT	570
	** **	
L78163	CTCAGGTGCTCATACGTTTGGAAAGAGCTCGGTGCAGTACATTCAATAACC	622
U41657	CTCAGGTGCTCATACGTTTGGAAAGAGCTCGGTGCAGTACATTCAATAACC	424
X90693	CTCGGTGCTCATACGTTTGGAAAGAGCTCGGTGCAGTACATTCAATAACC	633
X90694	CTCGGTGCTCATACGTTTGGAAAGAGCTCGGTGCAGTACATTCAATAACC	635
L36156	CTCGGTGCTCATACGTTTGGAAAGAGCTCGGTGCAGTACATTCAATAACC	614
X90692	CTCAGGTGCTCATACGTTTGGAAAGAGCTCGGTGCAGTACATTCAATAACC	620
	*** **	
L78163	GATTATACAACTTCAGCAACACTGGAAACCTGATCCAACTCTGAACACA	612
U41657	GATTATACAACTTCAGCAACACTGGAAACCTGATCCAACTCTGAACACA	466

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*****
L78163 TGTAATTTTGTGAA---TGGAGACTCGT-----TTGGATTAGC 1007
U41657 TGTAATTTTGTGAA---TGGAGACTCGT-----TTGGATTAGC 800
X90693 TGCAACTTTTGTTAATT-----CAAAATCAGCAGAACTTGGTCTTAT 1024
X90694 TGCAACTTTTGTGAACTTTGTGAACTCAAATTTCTGCAGAACTAGATTTAGC 1035
L36156 TGCAACTT-----TGTGAACTCAAATTTCTGCAGAACTAGATTTAGC 1005
X90692 TG-----TAATGCTGTGAATGGGAATTCCTC-----TGGATTGGC 1005
**
L73163 TAGTGTGGCGTCCAAAGATGCTAAACAAAAGCTTGTGTGCTCAATCTAAAT 1057
U41657 TAGTGTGGCGTCCAAAGATGCTAAACAAAAGCTTGTGTGCTCAATCTAAAT 850
X90693 CAATGTTCCTC---AGCAG--ATTCATCTG-AGGAGGGTATGGTTAG-- 1066
X90694 CACCATAGCATCCATAGTAG--AATCATTAG-AGGATGCTATTGCTAGTG 1082
L36156 CACCATAGCATCCATAGTAG--AATCATTAG-AGGATGGAATTGCTAGTG 1052
X90692 TACTGTAGTCACCAA---AG--AATCATCAG-AAGATGGAATGGCTAGCT 1049
*
L78163 AAACCAATAATTAATGGGATGTGCATGCTAGCTAGCATGTAAAGGCCAA 1107
U41657 AAACCAATAATTAATGGGATGTGCATGCTAGCTAGCATGTAAAGGCCAA 900
X90693 -----CTCAATGTAAA-TG-TAG 1082
X90694 TAATATAAATAAATTAG-----CGTAAATGCCACTTATTGAA-ATCTTG 1124
L36156 TAATATAAATAAATTAG-----CGAAATGCCACTTATTGAA-ATCTTG 1094
X90692 CATTCTAAAT--ATAAG-----CTTGGAATAATTGAAGAGGTTCTAT 1090
*
L78163 TTAGGTTGTAAACCTCTTTGCTAGCTATATTGAAATAAACCAAAGGAGTA 1157
U41657 TTAGGTTG-AAACCTCTTTGCTAGCTATATTGAAATAAACCAAAGGAGTA 949
X90693 T--GATTGGAAAGCACTAA--TAAATTAAGGAAGCTATAAC-----T 1119
X90694 T--GACTAGATGCCACTAA--TAAAT---AAGTTATAAC-----T 1157
L36156 T--GACTAGATCCCACTAA--TAAAT---AAGTTATAAC-----T 1127
X90692 A--ATTCTTGTGCATACATA--TATGTAATGTG----- 1118
*
L78163 GTGTGCATGTCAATTGGATTTCG-CATGTACCTCTTGGAAATAT----- 1200
U41657 GTGTGCATGTCAATTGGATTTCG-CATGTACCTCTTGGAAATATTATOTA 958
X90693 ATUCACATT-CATGTATGTGTGATAGTATTAGATGCTTTGTGAGCA 1166
X90694 AGGCACATTTCAATGCACTTGAAATTTCAATGCCCT-GTATATGAG----- 1200
L36156 ATUCACATTTCAATGCACTTGAAATTTCAATGCCCTGTATATTAGAGGAGC 1177
X90692 -----CATGTGTGTA--TTATGTTTTGTATGTTCTTCAGTTGATCA 1161
**
L78163 ----- 1200
U41657 ATAATTATTTGAATCTC-----AAAAAAAAAAAAAAAA 1031
X90693 AAAATCTTTTGGATTTC-----ATTTGAAGTCTTCT---- 1200
X90694 ----- 1200
L36156 TGT-TCCT-----C-----TTGTTATTATACTA--T 1200
X90692 UGGA-CTGTAGAGCTCCCTAATAATATTGTGTCAAAGT 1200

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FIGURE 3B

L78163	MGSMRLLVVALLCAPAMHAGFSVSY---AQLTPTFYRETCPNLPPIVFGV	47
U41657	-----	0
X90693	MNSLRAVALALCCIV--VVLGGLPFSSNAQLDPSFYRNTCPNVSSIVREV	48
X90694	MNSL---ATSMNCVLLVVLGGLPFSSPAQLSPTFYSKTCPTVSSIVSNV	47
L36156	M-----WCVLLVVLGGLPFSSDAQLSPTFYSKTCPTVSSIVSNV	40
X90692	MLGLSATA---PCCMVFLVIGGVPPS-NAQLDPSFTNS TCSNLD SIVRGV	46
L78163	IFDA3FTLPRIGASLRLHFHDCFVQGCDSVLLNNTDTIESEQDALPNI	97
U41657	-----FHD CFVQGCDSVLLNNTDTIESEQDALPNI	31
X90693	IRSVSKDPRMLASLVRLHFHDCFVQGCDA SVLQNKTDTVVSEQDA7PNR	98
X90694	LTVSKTDPRMLASLVRLHFHDCFVVGCDASVLLNNTATIVSEQAAPPNN	97
L36156	LTVSKTDPRMLASLVRLHFHDCFVVGCDASVLLNNTATIVSEQAAPPNN	90
X90692	LTVSQSDPRMLGSLIRLHFHDCFVQGCDA SILLND7ATIVSEQAAPPNN	96
	*****	
L78163	NSIRGLDVNDIKTAVENSCPDTVSCADILALAAEIASVLCGGPGWVPVL	147
U41657	NSIRGLDVNDIKTAVENSCPDTVSCADILALAAEIASVAGARRSGWVPVL	31
X90693	NSLRGLDVNQIKTAVEKACPN TVSCADILALSARLSSTIADGPDWKVPL	148
X90694	NSLRGLDVNQIKLAVEVPCPN TVSCADILALAAQASSVLAQGPPSWTVPL	147
L36156	NSLRGLDVNQIKTAVESACPN TVSCADILALA-QASSVLAQGPPSWT7PL	139
X90692	NSIRGLDVNQIKTAVENACPN TVSCADILALSARISSDLANGPTWQVPL	146
	*****	
L78163	GRRDSITANRTLANQNLPAPFFNLTLQKASFAVQGLNTLDLVTLGGHTF	197
U41657	GRRDSL TANRTLANQNLPAPFFNLTLQKASFAVQGLNTLDLVTLGGHTF	131
X90693	GRRDGLTANQLLANQNLPAPFNTTDQLKPAFAAQGLD TTDLVALSQAHTF	192
X90694	GRRDGLTANRTLANQNLPAPFNSLDQKAAFTAQGLNTTDLVALSQAHTF	197
L36156	GRRDGLTANRTLANQNLPAPFNSLDHLKLF LTAQGLITPVLVALSQAHTF	180
X90692	GRRDSL TANNSLAAQNLPAPTFNLTRKSNFDNQNI STTDLVALSQGHTI	196
	*****	
L78163	GRARCS TFINRLYNF SNTGNPDPTLNTTYLEVLRARCPQNA7GDNLTNLD	217
U41657	GRARCS TFINRLYNF SNTG L I N--LDTTYLEVLRARCPQNA7GDNLTNLD	179
X90693	GRAHCS LFVSRLYNFSGTGPDPPTLNTTYLQQLRTICPNGGPGTNLTNFD	218
X90694	GRAHCAQFVSRLYNFSGTGNPDPTLNTTYLQQLRTICPNGGPGTNLTNFD	247
L36156	GRAHCAQFVSRLYNFSGT3SPDPPTLNTTYLQQLRTICPNGGPGTNLTNFD	239
X90692	GRGQCRFFVDRLYNF SNTGNPDSTLNTTYLQTLQAICPNGGPGTNLTNLD	246
	*****	
L78163	LSTFDQFDNRYYSNLLQLNGLLQSDQELFSTPGADTIPIVNSFSSNQNTF	297
U41657	LSTFDQFDNRYYSNLLQLNGLLQSDQELFSTPGADTIPLSLA-SANQNTF	228
X90693	PTTPDKFDNRYYSNLQVKKGLLQSDQELFSTSGSDTISIVNKFATDQKAF	298
X90694	PTTPDKFDNRYYSNLQVKKGLLQSDQELFSTSGADTISIVNKFSTDONAF	297
L36156	PTTPDKFDNRYYSNLQVKKGLLQSDQELFSTSGADTISIVNKFSTDONAF	289
X90692	PTTPDTYLSNYYSNLQVKKGLFQEDQELFSRNGSDTISIVNSPANQTLF	296
	*****	
L78163	PSNFIIVSMIKMGNIGVLTGDEGEIRLQCNFVN-----GDSFGIASVAS-K	341
U41657	PSNFIIVSMIKMGNIGVLTGDEGEIRLQCNFVN-----GDSFGIASVAS-K	272
X90693	PESFRAAMIIMGNIGVLTGRQGBEIRKQCNFVN---SKSAELGLINVAS-A	344
X90694	PESFRAAMIIMGNIGVLTGTGKEIRKQCNFVN FVNSNSAPLOLATIASIV	347
L36156	PESFKAAMIIMGNIGVLTGTGKEIRKQCNFVN---SNEABLDLATIASIV	336

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X90692 FENFVASMIMGNIGVLTGSQGBIRTQCNVN-----GNSSGLATVVT-K 340  
 \* . . . . .

L78163	DAKQKLVAQSK	352
U41657	DAKQKLVAQSK	283
X90693	DSSEECMVSSM	355
X90694	ESLEDGLASVI	358
L36156	ESLEDGLASVI	347
X90692	ESSEDCMASSP	351

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FIGURE 4

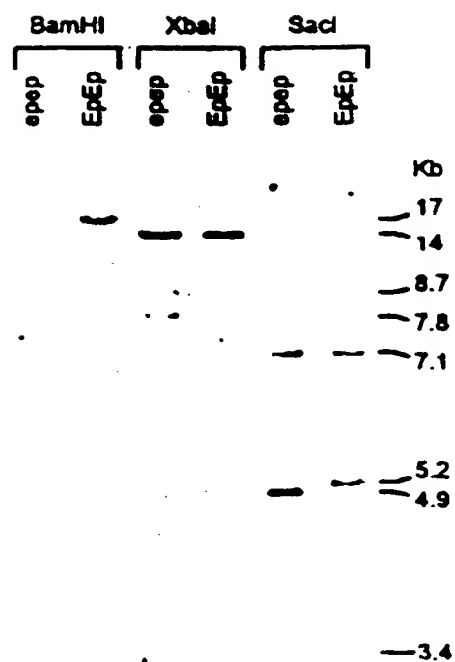
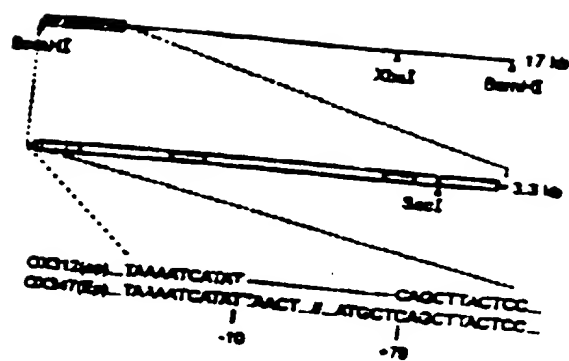
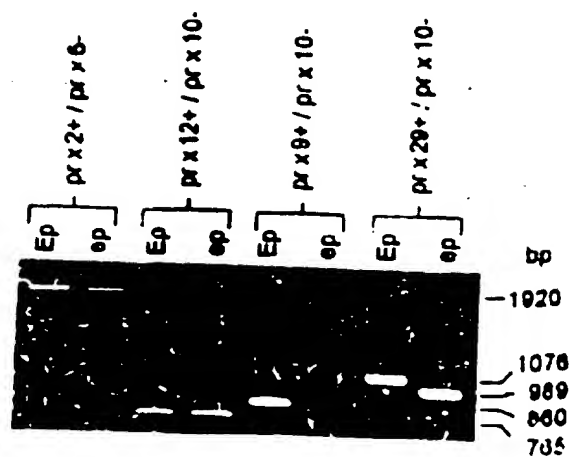
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FIGURE 5



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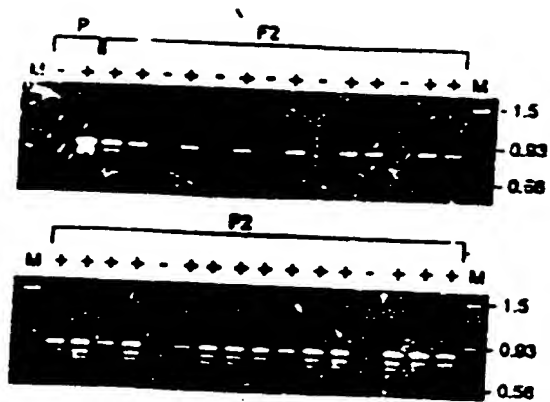
FIGURE 6



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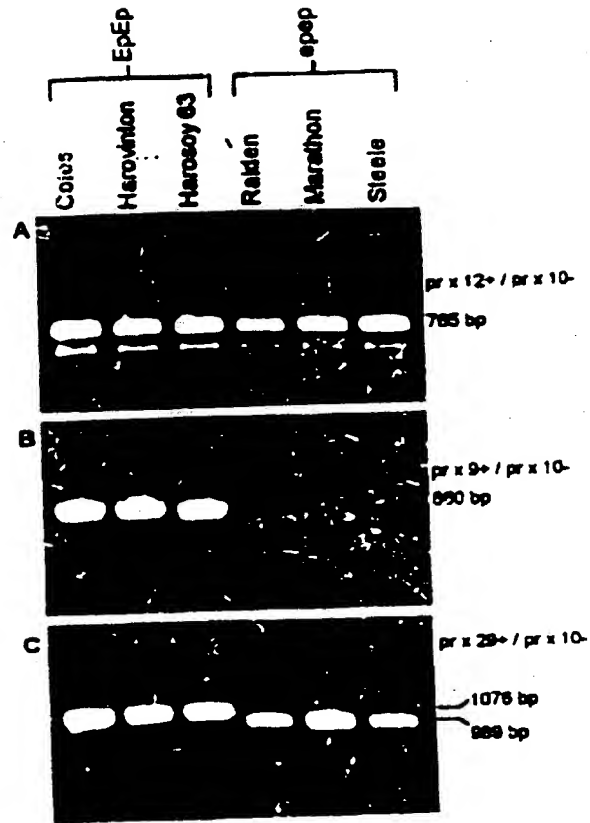


FIGURE 7



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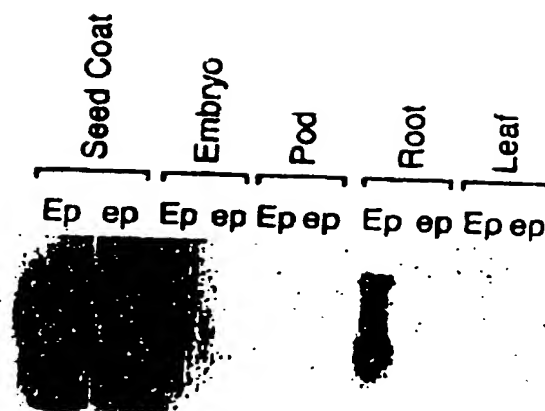
FIGURE 8



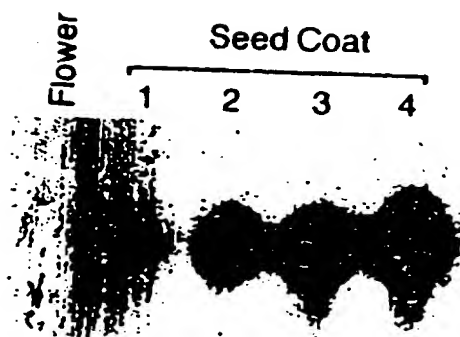
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FIGURE 9

A



B



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